# EFFECTS OF AN INTRODUCED PARASITIC NEST FLY ON ENDEMIC AVIAN HOSTS IN THE GALÁPAGOS ISLANDS

by

Sarah Ann Knutie

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biology

The University of Utah

December 2014

**The University of Utah Graduate School**

# STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Sarah Ann Knutie**

has been approved by the following supervisory committee members:

| | | |
|---|---|---|
| **Dale Clayton** | , Chair | **09/02/2014** Date Approved |
| **Denise Dearing** | , Member | **09/02/2014** Date Approved |
| **Franz Goller** | , Member | **09/02/2014** Date Approved |
| **Jeb Owen** | , Member | **09/02/2014** Date Approved |
| **Wayne Potts** | , Member | **09/02/2014** Date Approved |

and by **Denise Dearing** , Chair/Dean of

the Department/College/School of **Biology**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

Introduced parasites threaten native host populations in many parts of the world. For example, the introduced parasitic nest fly *Philornis downsi* has been implicated in the decline of Darwin's finch populations in the Galápagos Islands. Studies have just begun to rigorously test the question of why such parasites are successful, and how they devastate naïve host populations. In this dissertation, I examine the effects of *P. downsi* flies on two host species in the Galápagos; I explore the underlying mechanisms by which hosts are affected.

Using an experimental manipulation of *P. downsi,* I demonstrate that *P. downsi* reduces nestling survival of medium ground finches (*Geospiza fortis*), but not Galápagos mockingbirds (*Mimus parvulus*) at the same location. This suggests that mockingbirds are tolerant to the effects of *P. downsi*. Mockingbird nestlings appear to compensate for parasite damage by increasing their energy intake through increased begging and parental provisioning. Mockingbirds also appear to differentially express more genes in response to parasitism than finches. However, mockingbirds and finches express very few of the same genes in response to parasitism, suggesting that *P. downsi* has different effects on gene expression in these hosts.

I also investigated the effects of native parasitic flies *Philornis trinitensis* on hosts in Tobago to understand why *P. downsi* is successful in the Galápagos. I show that the effect of *P. trinitensis* on Tobago hosts is similar to the effect of *P. downsi* on Galápagos hosts. However, the prevalence of *P. trinitensis* in Tobago is lower than that in the

Galápagos, which may be because there are more enemies of the flies in Tobago compared to the Galápagos. Thus, introducing native predators of *P. downsi* to the Galápagos may be a promising approach to reducing the effect of *P. downsi* on finches. Until such predators are identified, I present evidence that self-fumigation by finches can be used as an effective stopgap approach to control *P. downsi.*

My work demonstrates that mockingbird reservoir hosts of *P. downsi* change their behavior to tolerate parasite damage. In turn, *P. downsi* can persist in the environment, regardless of future declines of vulnerable finch hosts, unless control methods are implemented.

TABLE OF CONTENTS

LIST OF TABLES

Tables

LIST OF FIGURES

Figures

# ACKNOWLEDGMENTS

The success of my dissertation would have never been possible without the support of many people in my life. I first must thank my advisor, Dale Clayton, for his mentorship throughout my dissertation work. Dale acknowledged and encouraged my passion for conservation ecology, despite the dominating theme of evolutionary biology with feral pigeons and lice in our lab. He pushed me to think critically about novel and relevant questions, which has undoubtedly made me a better scientist. Our 6-hour long meetings may have drained the life out of me at the time, but looking back, I can appreciate the amount of time he dedicated to help develop me as a scientist. Dale has always supported my research ambitions, even when my ideas were out of left field. I hope to continue our collaborations in the future and cannot thank him enough for his mentorship.

I also thank my committee, Jeb Owen, Franz Goller, Denise Dearing, and Wayne Potts, for constructive criticism and feedback, which greatly improved my dissertation. The committee, especially Wayne, encouraged me to return to the Galápagos for a final season to pursue the study for my final chapter, even when others doubted the project's success. I thank Jeb for taking a significant amount of his time to train me in his lab and teach me how to think more critically about the field of ecoimmunology. Franz encouraged me to join him in his local fieldwork and provided mentorship and inspiration that will stick with me through my career. Denise was a strong role model for me and

has also been essential in my decision to continue with a career in academia.

Lissy Coley, Ayako Yamaguchi, Nalini Nadkarni, and Sarah Bush are also strong women in the department who have inspired me to continue in academia. I thank them for their mentorship and our memorable conversations. Shannon Nielsen, Karen Zundal, Terry Merritt, and Renae Curtz offered enormous help with navigating graduated school, organizing rooms, and submitting grants.

The members of my lab, especially Scott Villa, were critical in surviving graduate school. Scott motivated me to succeed and has been a shoulder to cry on when I questioned why I was in graduate school. I also thank my other lab mates, including Sabrina McNew, Emily DiBlasi, Andrew Bartlow, Jen Koop, and Jessi Waite, for their support in the field and lab. Jordan Herman, Miriam Clayton, and Daniela Vargas contributed immensely to my dissertation work in the Galápagos and Tobago. I consider these women to be my "academic little sisters" and will be forever grateful for their hard work in the field.

My friends were essential in maintaining my sanity throughout graduate school. They offered distractions, commiseration, and unrelenting support. I thank the many biology graduate students of our department, but especially members of my cohort: Shannon Gaukler, Kevin Kohl, Kendra Chritz, and Carina Marón; these people have become my life-long friends and colleagues.

I am eternally grateful to my family. My parents sacrificed many things to support my career; I am indebted to them for their love, encouragement, and friendship. My sister and brother-in-law, as well as my extended family, also offered their endless support of my choice to pursue a PhD, even when they were not quite sure where this

path would lead to in the end. I especially appreciate the long-winded, open-minded conversations with my grandmother, Annabelle Smith. I feel lucky to have several strong female role models in my family.

I thank the Charles Darwin Foundation and Galápagos National Park for supporting my research. I also thank Goberth Cabrera for reliably driving us to our field site every morning, teaching me Spanish, and stopping for sandia and bananas after the most exhausting days. My dissertation was financially supported by a National Science Foundation grant to my advisor, Dale Clayton. I received additional funding through Frank M. Chapman Fund, American Ornithologist's Union, Society of Integrative and Comparative Biology (SICB), University of Utah Global Change and Sustainability Center, the University of Utah Department of Biology, and Rockethub Crowdfunding. I also received donations for my work from US Cotton, Floral Cotton, and Nature Valley.

My dissertation is dedicated to my master's advisor Dr. Maria E. Pererya, who passed away in August 2014. I will be forever grateful for her mentorship at the University of Tulsa.

# CHAPTER 1

# INTRODUCTION

## Background

Parasites influence fundamental aspects of the evolutionary ecology of their hosts (Anderson and May 1978, Anderson 1979). Hosts and parasites with a long-standing relationship have co-evolved adaptations that allow hosts to defend themselves and parasites to escape host defenses. These interactions can lead to relatively stable population dynamics between hosts and their parasites. In contrast, the introduction of parasites to a new location can have devastating effects on naïve host populations. One explanation is that naïve host populations lack effective defenses against such parasites (Daszak et al. 2000, Wikelski et al. 2004, Keesing et al. 2010).

Not all hosts are vulnerable to the effects of introduced parasites; in some cases, the fitness of hosts is clearly reduced, while the fitness of reservoir or tolerant hosts is relatively unaffected (Haydon et al. 2002, Schmid-Hempel 2011). Reservoir hosts can therefore provide a stable resource for the introduced parasite. For example, the Pacific chorus frog (*Pseudacris regilla*) in the Sierra Nevada of California is a reservoir for the chytrid fungus *Batrachochytrium dendrobatidis* (Reeder et al. 2012). Despite catastrophic declines of more vulnerable frog species, chorus frogs survive heavy fungal infections. Reservoir hosts, such as the chorus frog, can maintain large numbers of

parasites in the environment, even when vulnerable host populations are declining (Reeder et al. 2012). Therefore, parasite reservoirs are an indirect threat to populations of more vulnerable host species (Daszak et al. 2001, McCallum 2012).

Many reservoir host studies have focused on non-native hosts, such as introduced and domesticated animals (Roelke Parker et al. 1996, Laurenson et al. 2003, Prager et al. 2012). However, these hosts may be overstated as influential reservoir hosts, as in the case of the introduction of avian malaria parasites and their mosquito vectors to the Hawaiian Islands. Avian malaria is thought to have been partly responsible for the extinction of 17 endemic honeycreeper species (Atkinson and Lapointe 2009). For years, researchers thought that some introduced hosts were reservoirs for avian malaria (van Riper III and van Riper 1986). Instead, Atkinson et al. (2000) found that one endemic honeycreeper (*Hemignathus virens virens*) is relatively unaffected by the malaria parasite and likely maintains the parasite in the environment. Thus, the amakihi may be a reservoir host that amplifies the negative effect of malaria on more vulnerable and declining honeycreeper species (Atkinson and Lapointe 2009).

Defense strategies that differentiate vulnerable and reservoir hosts are even less understood. Host life history strategies may promote reservoir host populations. For example, parasites may affect reservoir host survival at an individual level, but if such hosts have a high turnover rate, then they would not suffer an overall effect of the parasite at the population level (*r*-selected species; Prager et al. 2012). Most studies suggest that reservoir hosts are able to tolerate parasite damage without reducing parasite load. However, few studies have identified specific mechanisms underlying host tolerance (Simms 2000, Read et al. 2008, Medzhitov et al. 2012, Sorci 2013). Such

studies have found that host behavioral and physiological responses can promote tolerance to the parasite under captive conditions. For example, tadpoles can behaviorally shunt the infection site of larval tremetodes from their heads to their tails to avoid an assault on their vital organs, but without affecting parasite abundance (Sears et al. 2013). Other hosts tolerate blood parasites by recovering red blood cells that are lost to the parasites (Råberg et al. 2009). However, field studies on tolerance mechanisms to parasites under natural conditions are rare (Reeder et al. 2012, Sorci 2013).

Abiotic and biotic factors can also influence the relationship between hosts and their parasites (Schmid-Hempel 2011). Another explanation for the success of introduced parasites in a new environment is the enemy release hypothesis (ERH; Keane and Crawley 2002, Liu and Stiling 2006). In this case, introduced parasites spread rapidly because they are liberated from their predators, which would regulate parasite population dynamics in their native range. The ERH is one of the most cited explanations for the success of introduced plant species, but only recently has this hypothesis been empirically tested (reviewed in Liu and Stiling 2006). The ERH has been suggested as important in animal host-parasite systems, but most of this research has focused on introduced hosts escaping their parasites in the new location rather than parasites escaping their enemies (Torchin et al. 2003, Torchin and Mitchell 2004). Indeed, introduced parasites are likely successful in a new location by escaping their enemies, but this concept has seldom been explored (Colautti et al. 2004).

Introduction of the parasitic nest fly *Philornis downsi* to the Galápagos Islands provides a rare opportunity to test classic hypotheses in parasite invasion ecology (Perkins et al. 2008). Adult flies (which are not parasitic) lay their eggs in the nests of

Darwin's finches and other land birds in the Galápagos. Once the fly eggs hatch, the larvae feed on the blood of nestlings and adult females when they sit on the nest. The native range of *P. downsi* includes Trinidad, Brazil, Argentina, and likely other areas in mainland South America (Dodge and Aitken 1968, Silvestri et al. 2011). The fly is also found on nearly all surveyed islands in the Galápagos (Wiedenfeld et al. 2007, Causton et al. 2013).

The effect of *P. downsi* on Darwin's finches has been studied extensively (Fessl et al. 2006, Huber 2008, O'Connor et al. 2010, 2013, Koop et al. 2011, 2013b, 2013a, Kleindorfer et al. 2014). *P. downsi* has been implicated in the decline of critically endangered species of finches, such as the medium tree finch and mangrove finch (Fessl et al. 2010, O'Connor et al. 2010). In some years, 100% of Darwin's finch nests at a given location fail to produce fledglings as a direct result of *P. downsi* (Koop et al. 2013a, O'Connor et al. 2013). Finches clearly do not have effective defenses against the parasite (Koop et al. 2013a). Kleindorfer et al. (2014) suggest that over the past decade, finch mortality has increased and age at mortality has decreased due to earlier infestation during the nestling period (although this trend may be related to climate variation). However, the effect of *P. downsi* on non-finch species has not been tested. Indeed, information on other hosts and possible defenses against *P. downsi* is particularly useful to determine: 1) if there is variation in the effects on host fitness, 2) if birds have the potential to evolve effective defenses against *P. downsi*, and 3) if there are some birds that are tolerant to the effects of *P. downsi*.

Several methods for controlling *P. downsi* populations are currently being considered or tested (Causton et al. 2013). Reducing the effect of *P. downsi* by lowering

the parasite population may delay or eliminate extinction risk for the finches. Traps designed to capture adult flies offer a low-cost, low-maintenance option for reducing populations. However, attempts to capture adult flies using food bait (e.g., fruits, water, and syrups) have only been minimally successful (Causton et al. 2013). Augmentative biological control with native predators of *P. downsi* may also provide a long-term solution because this method can be used over a large area. Identifying native predators of *Philornis* is still necessary to determine whether biological control is appropriate for the Galápagos.

The most effective method of *P. downsi* control, to date, is the direct application of permethrin in nests, which is extremely labor intensive (Koop et al. 2013b, 2013a, O'Connor et al. 2013). In theory, direct fumigation is ideal for small populations of finches, such as the mangrove finch. However, mangrove finches nest high in mangrove trees (13 meters on average), which makes many nests nearly impossible to reach. Feasible methods to combat P. downsi have not yet been developed, and thus, are a high priority in the Galápagos.

My dissertation examines the effects of *P. downsi* on two species of endemic avian hosts in the Galápagos. I use an experimental approach to determine whether medium ground finches (*Geospiza fortis*) are affected by *P. downsi* because mothers are 'stressed' by the parasite (Chapter 2). Then, I compare the effects of *P. downsi* on finches to another host species nesting in the area, the Galápagos mockingbird (*Mimus parvulus*; Chapter 3). I compare mechanisms underlying why finches and mockingbirds are differentially affected by the parasite (Chapter 3). I also compare the effect of *P. downsi* on finch and mockingbird gene expression (Chapter 4). I then compare the

effects of native *Philornis* flies on hosts in Tobago and I survey potential predators of *Philornis* in their native range (Chapter 5). Finally, I test the effectiveness of "self-fumigation" to control *P. downsi* in the nests of finches (Chapter 6).

## Chapter Summaries

### Chapter 2: Experimental test of the effect of introduced hematophagous flies on corticosterone levels of breeding Darwin's finches

Chapter 2 examines the effect of an invasive parasite on corticosterone concentrations of a common species of Darwin's finch, the medium ground finch. High stress levels as a result of *P. downsi* could reduce the ability of females to invest in offspring, thus decreasing their reproductive success. Thus, the effect of *P. downsi* on host reproductive success could be mediated by stress responses in breeding female finches. To test this hypothesis, we experimentally manipulated the abundance of *P. downsi* in nests, then measured baseline and acute stress-induced corticosterone levels, body condition, and hematocrit (red blood cell content). Acute stress-induced corticosterone levels increased over baseline levels, but this response did not differ significantly with parasite treatment. There was also no significant difference in the body condition or hematocrit of females from parasitized versus nonparasitized nests. The results suggest that the lower reproductive success of females from parasitized nests is not mediated by a physiological stress response.

### Chapter 3: Galápagos mockingbirds are reservoir hosts for introduced parasites that threaten Darwin's finches

Chapter 3 compares the effect of *P. downsi* on Galápagos mockingbirds and medium ground finches. We experimentally manipulated *P. downsi* abundance in the

nests of mockingbirds and medium ground finches to compare the effects of the parasite on host reproductive fitness. We also examined host behavioral responses. We found that although the two host species had similar numbers of parasites per gram of host body mass and lost significant amounts of blood hemoglobin to the parasite, *P. downsi* decreased nestling growth and survival of finches only. Among finch nestlings, plasma glucose levels, begging, and parental provisioning did not change in response to *P. downsi*. In contrast, mockingbird nestlings increased energy intake in response to *P. downsi*, with parasitized mockingbird nestlings begging more than nonparasitized nestlings. Greater begging was related to increased parental provisioning, which may have compensated for parasite damage. Our study suggests that finches are negatively affected by *P. downsi* because they do not have an effective behavioral feedback system to compensate for energy lost to the parasite. Furthermore, mockingbirds are tolerant to the effects of *P. downsi* and amplify the effects of the parasite on Darwin's finches and other hosts in the Galápagos.

## Chapter 4: The effect of introduced parasitic nest flies on gene expression of Galápagos hosts

Chapter 4 explores the potential mechanisms that may explain differences in the effect of the parasite on hosts. We compared gene expression in erythrocytes of parasitized and nonparasitized nestling finches and mockingbirds. We found that a significant number of genes were expressed differentially in finches and mockingbirds when exposed to the parasite. More genes were expressed differentially in parasitized mockingbirds compared to parasitized finches. However, few of these genes were the same in the two species, suggesting that the genetic basis of the effect of the parasite may

be host species specific. Parasitized hosts did not demonstrate differential expression of genes related to the immune response. Instead, *P. downsi* affected a significant number of host genes related to other physiological processes, such as metabolism. Notably, parasitized finches showed significantly more expression in genes related to DNA repair compared to mockingbirds, suggesting that more DNA damage occurred in finches, with downstream effects on development and survival. Our study suggests that the introduced parasitic nest fly has a significant, but species-specific, effect on gene expression in the nestlings of the two different host species in the Galápagos.

### Chapter 5: Why are introduced parasites successful? Comparing the ecology of parasites in their native and novel ranges

Chapter 5 explores the ecology of native *Philornis* flies and native host species in Tobago. Specifically, the goal of this chapter was to examine the effect of native *Philornis* nest flies on native hosts in Tobago compared to the effects of introduced *P. downsi* parasitizing novel hosts in the Galápagos Islands. In Tobago, we experimentally manipulated native *Philornis trinitensis* abundance in nests of black-faced grassquits (*Tiaris bicolor*) and tropical mockingbirds (*Mimus gilvis*) to compare the effects of the parasite on host reproductive fitness. These Tobago host species are closely related to the Galápagos host species. We used the same methods to test for the effects of *P. downsi* on Galápagos hosts. Finally, we determined the effectiveness of host immune response against *Philornis* and surveyed potential predators of *Philornis* in bird nests. The number of parasites per gram of nestling mass was similar between related Tobago and Galápagos hosts. *Philornis* had a significant negative effect on grassquits and finches, but relatively little effect on either species of mockingbird. Fewer nests had parasites in

Tobago than the Galápagos; thus, the population-wide effect of *Philornis* was lower in Tobago than the Galápagos. Neither Tobago nor Galápagos nestlings had an effective immune response to reduce parasite load. Parasitoid wasps and ants were not found in the Galápagos. In Tobago, by contrast, 30% of nests had parasitoid wasps and 50% of nests had ants; parasite prevalence also tended to decrease throughout the season. This suggests that top down effects of wasps and ants on parasitic flies may reduce fly prevalence. Parasitic flies may be more prevalent in the Galápagos because they have escaped their natural enemies.

### Chapter 6: Darwin's finches combat introduced parasitic nest flies with fumigated cotton

Chapter 6 tests a potential method of controlling *P. downsi* in Darwin's finch nests. We show that Darwin's finches can be encouraged to 'self-fumigate' nests with cotton fibers that have been treated with permethrin. Nests with permethrin-treated cotton had half as many *P. downsi* as control nests, and nests containing at least one gram of cotton were virtually parasite-free. Nests directly fumigated with permethrin had fewer parasites and fledged more offspring than nests treated with water. The results from this study show that self-fumigation can be used to mitigate the effect of nest flies on Darwin's finches and perhaps other host species.

## References

Anderson, R. M. 1979. Parasite pathogenicity and the depression of host population equilibria. Nature 279:150–152.

Anderson, R. M., and R. M. May. 1978. Regulation and stability of host-parasite population interactions: I. Regulatory processes. Journal of Animal Ecology 47:219–247.

Atkinson, C. T., and D. A. Lapointe. 2009. Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. Journal of Avian Medicine and Surgery 23:53–63.

Causton, C., F. Cunninghame, and W. Tapia. 2013. Management of the avian parasite *Philornis downsi* in the Galápagos Islands: A collaborative and strategic action plan. Galapagos Reports. Galápagos National Park.

Colautti, R. I., A. Ricciardi, I. a. Grigorovich, and H. J. MacIsaac. 2004. Is invasion success explained by the enemy release hypothesis? Ecology Letters 7:721–733.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443–449.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica 78:103–116.

Dodge, H. R., and T. H. G. Aitken. 1968. *Philornis* Flies from Trinidad (Diptera: Muscidae). Journal of the Kansas Entomological Society 41:134–154.

Fessl, B., B. J. Sinclair, and S. Kleindorfer. 2006. The life-cycle of *Philornis downsi* (Diptera: Muscidae) parasitizing Darwin's finches and its impacts on nestling survival. Parasitology 133:739–747.

Fessl, B., G. H. Young, R. P. Young, J. Rodríguez-Matamoros, M. Dvorak, S. Tebbich, and J. E. Fa. 2010. How to save the rarest Darwin's finch from extinction: the mangrove finch on Isabela Island. Philosophical Transactions of the Royal Society of London B 365:1019–1030.

Haydon, D. T., S. Cleaveland, L. H. Taylor, and M. K. Laurenson. 2002. Identifying reservoirs of infection: a conceptual and practical challenge. Emerging Infectious Diseases 8:1468–1473.

Huber, S. K. 2008. Effects of the introduced parasite *Philornis downsi* on nestling growth and mortality in the medium ground finch (*Geospiza fortis*). Biological Conservation 141:601–609.

Keane, R. M., and M. J. Crawley. 2002. Exotic plant invasions and the enemy release hypothesis. Trends in Ecology and Evolution 17:164–170.

Keesing, F., L. K. Belden, P. Daszak, A. Dobson, C. D. Harvell, R. D. Holt, P. Hudson, A. Jolles, K. E. Jones, C. E. Mitchell, S. S. Myers, T. Bogich, and R. S. Ostfeld. 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468:647–652.

Kleindorfer, S., K. J. Peters, G. Custance, R. Y. Dudaniec, and J. A. O'Connor. 2014. Changes in *Philornis* infestation behavior threaten Darwin's finch survival. Current Zoology 60:542–550.

Koop, J. A. H., J. P. Owen, S. A. Knutie, M. A. Aguilar, and D. H. Clayton. 2013a. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecology and Evolution 3:2514–2523.

Koop, J. A., S. K. Huber, S. M. Laverty, and D. H. Clayton. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PloS One 6:e19706.

Koop, J. A., C. LeBohec, and D. H. Clayton. 2013b. Dry year does not reduce invasive parasitic fly prevalence or abundance in Darwin's finch nests. Reports in Parasitology 3:11–17.

Laurenson, M. K., R. A. Norman, L. Gilbert, H. W. Reid, and P. J. Hudson. 2003. Identifying disease reservoirs in complex systems: mountain hares as reservoirs of ticks and louping-ill virus, pathogens of red grouse. Journal of Animal Ecology 72:177–185.

Liu, H., and P. Stiling. 2006. Testing the enemy release hypothesis: a review and meta-analysis. Biological Invasions 8:1535–1545.

McCallum, H. 2012. Disease and the dynamics of extinction. Philosophical Transactions of the Royal Society of London B: Biological Sciences 367:2828–2839.

Medzhitov, R., D. Schneider, and M. Soares. 2012. Disease tolerance as a defense strategy. Science 335:936–941.

O'Connor, J. A., J. Robertson, and S. Kleindorfer. 2013. Darwin's finch begging intensity does not honestly signal need in parasitised nest. Ethology 119:1–10.

O'Connor, J. A., F. J. Sulloway, J. Robertson, and S. Kleindorfer. 2010. *Philornis downsi* parasitism is the primary cause of nestling mortality in the critically endangered Darwin's medium tree finch (*Camarhynchus pauper*). Biodiversity and Conservation 19:853–866.

Perkins, S. E., A. Sonia, O. Bjornstad, J. J. Burdon, K. Clay, L. Gomez-Aparicio, J. M. Jeschke, P. T. J. Johnson, K. D. Lafferty, C. M. Malmstrom, P. Martin, A. Power, D. L. Strayer, P. H. Thrall, and M. Uriarte. 2008. Invasion biology and parasitic infections. Pages 179–204 *in* R. S. Ostfeld, F. Keesing, and V. T. Eviner, editors. Infectious Disease Ecology: Effects of Ecosystems on Disease and of Disease on Ecosystems. Princeton University Press, Princeton, New Jersey.

Prager, K., J. Mazet, E. Dubovi, L. Frank, L. Munson, A. Wagner, and R. Woodroffe. 2012. Rabies virus and canine distemper virus in wild and domestic carnivores in northern Kenya: are domestic dogs the reservoir? Ecohealth 9:483–498.

Råberg, L., A. L. Graham, and A. F. Read. 2009. Decomposing health: tolerance and resistance to parasites in animals. Philosophical Transactions of the Royal Society of London B: Biological Sciences 364:37–49.

Read, A. F., A. L. Graham, and L. Råberg. 2008. Animal defenses against infectious agents: is damage control more important than pathogen control. PLoS Biology 6:e4.

Reeder, N. M. M., A. P. Pessier, and V. T. Vredenburg. 2012. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PloS One 7:e33567.

Van Riper III, C., and S. van Riper. 1986. The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecological Monographs 56:327–344.

Roelke Parker, M. E., L. Munson, C. Packer, R. Kock, S. Cleaveland, M. Carpenter, S. J. O'Brien, A. Pospischil, R. Hofman Lehmann, H. Lutz, G. L. M. Mwamengele, M. N. Mgasa, G. A. Machange, B. A. Summers, and M. J. G. Appel. 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature 379:441–445.

Schmid-Hempel, P. 2011. Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics. Page 544. Oxford University Press, Oxford.

Sears, B. F., P. W. Snyder, and J. R. Rohr. 2013. Infection deflection: hosts control parasite location with behaviour to improve tolerance. Proceedings of the Royal Society B: Biological Sciences 280.

Silvestri, L., L. R. Antoniazzi, M. S. Couri, L. D. Monje, and P. M. Beldomenico. 2011. First record of the avian ectoparasite *Philornis downsi* Dodge & Aitken, 1968 (Diptera: Muscidae) in Argentina. Systematic Parasitology 80:137–140.

Simms, E. L. 2000. Defining tolerance as a norm of reaction. Evolutionary Ecology 14:563–570.

Sorci, G. 2013. Immunity, resistance and tolerance in bird-parasite interactions. Parasite Immunology 35:350–361.

Torchin, M. E., K. D. Lafferty, A. P. Dobson, V. J. Mckenzie, and A. M. Kuris. 2003. Introduced species and their missing parasites. Nature 421:628–630.

Torchin, M. E., and C. E. Mitchell. 2004. Parasites, pathogens, and invasions by plants and animals. Frontiers in Ecology and the Envionment 2:183–190.

Wiedenfeld, D. A., G. A. Jiménez U., B. Fessl, S. Kleindorfer, and J. C. Valarezo. 2007. Distribution of the introduced parasitic fly *Philornis downsi* (Diptera, Muscidae) in the Galápagos Islands. Pacific Conservation Biology 13:14–19.

Wikelski, M., J. Foufopoulos, H. Vargas, and H. Snell. 2004. Galápagos birds and diseases: invasive pathogens as threats for island species. Ecology and Society 9:online: http://www.ecologyandsociety.org/vol9/iss1.

# CHAPTER 2

# EXPERIMENTAL TEST OF THE EFFECT OF INTRODUCED HEMATOPHAGOUS FLIES ON CORTICOSTERONE LEVELS OF BREEDING DARWIN'S FINCHES

Printed with permission from:
Knutie, S.K., J.A.H. Koop, S.S. French, and D.H. Clayton. 2013. Experimental test of the effect of introduced hematophagous flies on corticosterone levels of breeding Darwin's finches. General and Comparative Endocrinology 193: 68-71, doi: 10.1016/j.ygcen.2013.07.009.

General and Comparative Endocrinology 193 (2013) 68–71

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

# Experimental test of the effect of introduced hematophagous flies on corticosterone levels of breeding Darwin's finches

Sarah A. Knutie [a,*,1], Jennifer A.H. Koop [a,1,2], Susannah S. French [b], Dale H. Clayton [a]

[a] Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA
[b] Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322, USA

ARTICLE INFO

*Article history:*
Received 1 November 2012
Revised 12 June 2013
Accepted 15 July 2013
Available online 24 July 2013

*Keywords:*
Condition
*Geospiza fortis*
Hematocrit
Invasive
*Philornis downsi*

ABSTRACT

Parasites can negatively affect the evolutionary fitness of their hosts by eliciting physiological stress responses. Parasite-induced stress can be monitored by measuring changes in the adrenal steroid hormone corticosterone. We examined the effect of an invasive parasite on the corticosterone concentrations of a common species of Darwin's finch, the medium ground finch (*Geospiza fortis*). *Philornis downsi* (Diptera: Muscidae) is a parasitic nest fly recently introduced to the Galapagos Islands, where it feeds on the blood of nestlings and breeding adult female finches. Previous work shows that *P. downsi* significantly reduces the reproductive success of several species of finches. We predicted that the effect of *P. downsi* on host reproductive success is mediated by stress responses in breeding female finches. High stress levels could reduce the ability of females to invest in offspring, thus decreasing their reproductive success. To test this hypothesis, we experimentally manipulated the abundance of *P. downsi* in nests, then measured baseline and acute stress-induced corticosterone levels, body condition, and hematocrit (red blood cell content). Acute stress-induced corticosterone levels increased over baseline levels, but this response did not differ significantly with parasite treatment. There was also no significant difference in the body condition or hematocrit of females from parasitized versus non-parasitized nests. Our results suggest that the lower reproductive success of females from parasitized nests is not mediated by a physiological stress response.



## 1. Introduction

An animal's ability to cope physiologically with environmental stressors is an important component of its evolutionary fitness (Breuner et al., 2008; Johnstone et al., 2012; Siegel, 1980). One mechanism that mediates this process is the regulation of glucocorticoids, such as corticosterone, through activation of the hypothalamic–pituitary–adrenal axis (HPA-axis) (Sapolsky et al., 2000). Short-term elevations in corticosterone can trigger adaptive responses, such as energy mobilization, activation of the immune system, increased delivery of oxygen to tissues, and night restfulness (Sapolsky et al., 2000; Wingfield et al., 1998). These responses act to help an organism recover from sources of stress and maintain homeostasis (McEwen and Wingfield, 2003; Wingfield and Kitaysky, 2002). However, long-term elevations in corticosterone can have detrimental effects on an organism's survival and reproductive success by over-depleting fat stores, reducing parental investment in offspring, or compromising host immune responses (Sapolsky et al., 2000; Silverin, 1986; Tsigos and Chrousos, 2002).

Parasites are common stressors faced by most organisms, including birds (Brown et al., 2005; Siegel, 1980). Several studies show that parasitized birds maintain higher levels of both baseline and acute stress-induced corticosterone than non-parasitized birds (Boughton et al., 2006; Brown et al., 2005; Raouf et al., 2006). Nest parasites – those which reside primarily in the nest material – can directly affect both nestlings and breeding adult birds (Clayton and Tompkins, 1994; Marshall, 1981). Indirect effects of parasitism, mediated through the stress response of adult birds, can also exacerbate the direct effects of parasites. For example, increased glucocorticoid activity by parasitized adults may alter parental investment in nestlings, in exchange for self-preservation (Wingfield and Silverin, 1986). Elevated corticosterone levels in breeding birds have been linked to: (1) delays in returning to the breeding grounds (Breuner and Hahn, 2003); (2) less time devoted to guarding nests (Kitaysky et al., 2001; Wingfield and Silverin, 1986); (3) less time feeding nestlings (Silverin, 1986); and (4) an increase in nest abandonment (Love et al., 2004; Silverin, 1986). Thus, while elevations in corticosterone can help to preserve adults, the consequences for nestlings may be severe, reducing adult reproductive success.

* Corresponding author. Fax: +1 801 581 4668.
*E-mail address:* saknutie@gmail.com (S.A. Knutie).
[1] These authors contributed equally to this work.
[2] Current address: Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA.

0016-6480/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2013.07.009

In the Galapagos Islands of Ecuador, an introduced parasitic fly, *Philornis downsi* (Diptera: Muscidae), affects several species of land birds, including Darwin's finches (Fessl and Tebbich, 2002). Adult flies, which are non-parasitic, feed on decaying, organic matter. Female flies lay their eggs in the nests of birds, or in the nares (nostrils) of nestlings (Fessl et al., 2006). Once the fly eggs hatch, the larvae are hematophagous parasites that feed on the blood of both nestlings and adult female birds when they sit on the nest (Huber et al., 2010). Adult females incubate the eggs and brood the offspring. Adult males do not sit on the nest and, therefore, do not appear to come into contact with the parasites (Huber et al., 2010; Koop et al., 2011). *P. downsi* was first documented in the Galapagos Islands in 1964 (Causton et al., 2006); however, it was not until 1997 that the fly was observed in large numbers in nests (Fessl et al., 2001; Fessl and Tebbich, 2002). *P. downsi* has a significant negative effect on the growth rates and fledging success of medium ground finches (*Geospiza fortis*) (Koop et al., 2011). However, little is known regarding the effect of *P. downsi* on adult birds, or whether such effects contribute to observed decreases in reproductive success.

We tested whether *P. downsi* causes an increase in the baseline or acute stress responses of breeding adult female medium ground finches. We also measured the effect of the parasite on various aspects of female condition. We experimentally manipulated *P. downsi* abundance in the nests of medium ground finches to test the effect of the parasite on adult female corticosterone concentration, body condition, and blood loss. We predicted that parasitized females would have higher levels of baseline corticosterone than non-parasitized females. We also predicted that parasitized females would exhibit a higher acute corticosterone response to handling induced stress. Assuming that corticosterone is involved in mobilizing sources of stored energy, such as fat and glucose (Wingfield et al., 1998), we predicted that an increase in glucocorticoid levels would coincide with a reduction in body condition. Finally, we predicted that parasitized females would have lower hematocrit than non-parasitized females. Reduced hematocrit (% red blood cells/total blood volume) is an indicator of blood loss in birds (Olaymeni 2009; Palmer et al., 1979).

## 2. Methods

### 2.1. Study species and site

The study was conducted January–April, 2010 at El Garrapatero on Santa Cruz Island, Galapagos. The field site is a 1.5 km × 1.5 km area located in the arid coastal zone. Medium ground finches are abundant at this site (Huber, 2008), where they nest in endemic tree cacti (*Opuntia echios gigantea*) 1.5–4 m above the ground. Finch clutch sizes range from 2–5 eggs, and the eggs are incubated for approximately 14 days (Grant, 1999). Nestlings normally fledge 10–14 days after hatching.

### 2.2. Manipulation of parasite abundance

Active nests were checked every other day between 600 and 1100 h throughout the study. When the first nestling hatched, the nest was randomly assigned to either an experimental or control group. Nestlings were temporarily removed while experimental nests ($n = 15$) were sprayed with a 1% permethrin solution (hereafter, fumigated nests) and control nests ($n = 15$) were sprayed with water (hereafter, sham-fumigated). After all of the nestlings in a nest had fledged or died, the nest was collected and sealed in a plastic bag; medium ground finches do not reuse nests (Grant 1999). Nests were dissected within 8 h of collection and *P. downsi* larvae, pupae, and eclosed pupal cases were counted. First instar larvae were not included in tallies of parasite abundance because they are too small to see reliably in the nest material (Koop et al., 2011). Total parasite abundance was calculated as the sum of all second and third instar larvae, pupae, and eclosed pupal cases found in a nest.

### 2.3. Field monitoring procedures

Adult birds and nests were monitored to determine their reproductive stage. When nestlings were 3–6 days old, a mist net was placed in the nest territory to catch the attending adult female. This time period was chosen to minimize sample loss due to the failure of parasitized nests, which often happens less than a week after hatching of the first egg (Koop et al., 2013). Once opened, nets were under constant surveillance. Netted birds were removed from the net and a blood sample was taken within 3 min of capture to assess baseline corticosterone levels. For each bird, a sterile 28-gauge needle was used to puncture the brachial (alar) vein, and the blood sample (<75 μL) was collected into a heparinized microhematocrit tube. Critoseal-sealed tubes were held on ice until centrifugation, which took place within 6 h of collection. Capillary tubes were spun at 8000 rpm for 10 min in a centrifuge. Hematocrit was measured in the capillary tube from the first blood sample with digital calipers to estimate the volume of packed red blood cells in relation to total blood volume. Blood and plasma were transferred and stored in separate 0.5 mL microcentrifuge vials at −20 °C until the end of the field season. Samples were then transported to the University of Utah and stored at −80 °C until processing for hormones.

Following collection of the first blood sample, each female finch was fitted with a numbered monel metal band and three plastic color bands for identification. Birds were weighed to the nearest 0.1 g and tarsal length was measured in triplicate. Body condition was estimated with a scaled mass index (SMI), calculated using body mass and tarsus length, as described by Peig and Green (2009). Following banding, each bird was placed in an individual cloth bag and another blood sample (<75 μL) was drawn 15 min after capture. The second blood sample was used to monitor changes in corticosterone (stress-induced corticosterone) upon exposure to an acute stressor, following the method of Wingfield et al. (1982). Because of the hot climate at our field site, which poses a danger to the health of captive birds, we released birds within 20 min of capture.

### 2.4. Radioimmunoassay protocol

Plasma samples were assayed in duplicate for corticosterone (antibody from Fitzgerald #20-CR45) using a previously described protocol (French et al., 2010). Briefly, samples were extracted using a 30% ethyl acetate/isooctane mixture. Corticosterone was separated from the sample using column chromatography (50% ethyl acetate/isooctane elution). The ethyl acetate/isooctane phase was separated, dried, and re-suspended in PBS buffer. For each sample we used an aliquot of the re-suspended fractions to measure individual recoveries following extraction and chromatography. These recoveries were used to adjust the final sample concentration values to account for any losses during these procedures. The coefficient of variation for corticosterone was 13.5% and the average minimum detectable value was 0.3 ng/mL. Two females (both from sham-fumigated nests) had baseline corticosterone values that fell below the standard curve for the assay (outside the range of detectable values); these birds were excluded from further analyses. Because plasma volumes were limited, these samples could not be re-run at different dilutions.

### 2.5. Statistical analysis

Statistical analyses were done in Prism® v.5.0b (GraphPad Software, Inc.). All relevant parameters were tested for normality using a D'Agostino and Pearson omnibus normality test. Parasite abundance was compared between treatments with a Mann–Whitney *U* test. A two-way ANOVA was used to compare baseline and stress-induced corticosterone concentrations between parasite treatments. Body condition and hematocrit were compared between parasite treatments with separate two-tailed *t*-tests.

## 3. Results

The experimental manipulation of nests was successful. Sham-fumigated nests had a mean ± SE of 44.73 ± 6.30 parasites/nest (lower and upper 95% confidence intervals of the mean (95% CI: 31.23–58.24), whereas fumigated nests had a mean of 0.27 ± 0.27 parasites/nest (95% CI: −0.31 to 0.84) (Fig. 1; Mann–Whitney, $U = 0.00$, $P < 0.0001$). Fourteen of the 15 fumigated nests were parasite free; the remaining nest, which experienced heavy rain soon after being treated, had four fly larvae. The female finch from this nest was included in all analyses. In the fifteen sham-fumigated nests, parasite abundance ranged from 5 to 79 flies per nest.

There was a significant effect of stress on corticosterone concentration: stress-induced corticosterone was significantly higher than baseline corticosterone (Fig. 2; two-way ANOVA, $F_{1,50} = 131.5$, $P < 0.0001$). However, there was no significant effect of parasite treatment on corticosterone ($F_{1,50} = 0.0001$, $P = 0.99$), nor was there a significant interaction between treatment and time ($F_{1,50} = 0.12$, $P = 0.73$). Baseline corticosterone values were as follows: fumigated: $n = 13$, mean ± SE = 13.29 ± 0.82 ng/mL, 95% CI: 11.51–15.08; sham-fumigated: $n = 11$, mean ± SE = 11.89 ± 0.93 ng/mL, 95% CI: 9.81–13.96. Stress-induced corticosterone values were as follows: fumigated $n = 15$, mean ± SE = 58.54 ± 4.90 ng/mL, 95% CI: 48.03–69.05; sham-fumigated $n = 15$, mean ± SE = 57.22 ± 4.94 ng/mL, 95% CI: 46.62–67.81.

Body condition (estimated by SMI) did not differ significantly between females from fumigated ($n = 14$, mean ± SE = 21.8 ± 0.9 g, 95% CI: 19.9–23.7) and sham-fumigated nests ($n = 12$, mean ± SE = 23.3 ± 1.0 g, 95% CI: 21.1–25.5; two-tailed *t*-test, $t = 1.15$, $df = 24$, $P = 0.26$). Likewise, hematocrit did not differ significantly between treatments (fumigated: $n = 13$, mean ± SE = 47.08, 95% CI: 44.74–49.41 ± 1.10; sham-fumigated: $n = 14$, mean ± SE = 47.57, 95% CI: 45.78–49.36 ± 0.83; two-tailed *t*-test, $t = 0.37$, $df = 25$, $P = 0.72$). Across both treatments, baseline corticosterone did not correlate significantly either with body condition (Spearman, $r = -0.20$, $P = 0.38$) or hematocrit ($r = -0.16$, $P = 0.49$).

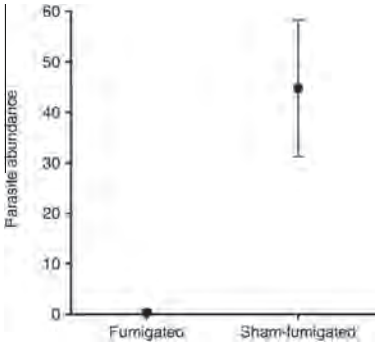


**Fig. 1.** Comparison of the mean (95% CI) number of *P. downsi* in fumigated ($n = 15$) and sham-fumigated ($n = 15$) nests.

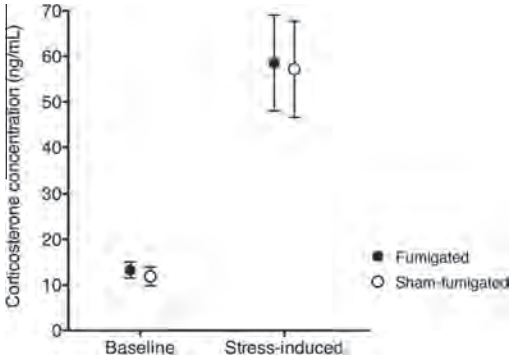


**Fig. 2.** Mean plasma corticosterone levels (95% CI) in female medium ground finches from fumigated and sham-fumigated nests. Baseline measurements were taken within 3 min of capture; stress-induced measurements were taken 15 min after capture.

## 4. Discussion

To our knowledge, this is the first study to examine the stress response of Darwin's finches in relation to parasites. We found that female finches are capable of a functional stress response, as indicated by significantly higher stress-induced corticosterone levels than baseline levels. We predicted that females at parasitized nests would have higher levels of baseline and stress-induced corticosterone than females at non-parasitized nests. However, there was no significant difference between parasite treatments, indicating that parasitism by *P. downsi* does not alter corticosterone levels in adult female finches, at least over the time interval of our study.

We also investigated whether *P. downsi* affects female body condition and hematocrit, which are known correlates of corticosterone response (Kitaysky et al., 1999; Schoech et al., 1997; Sockman and Schwabl, 2001). *P. downsi* had a significant negative effect on the reproductive success of birds in this study; see Koop et al. (2013) for details. However, neither the body condition nor hematocrit of adult females differed significantly between parasite treatments. These results suggest that female medium ground finches did not suffer direct adverse effects of parasitism by *P. downsi*.

There are several possible explanations for why we did not observe a relationship between parasitism and corticosterone. The simplest explanation is that*P. downsi* is not a significant stressor for adult female birds. While previous studies suggest that female finches are bitten by *P. downsi* (Huber et al., 2010; Koop et al., 2013), the frequency with which this occurs is unknown. Our study did not find a significant difference in hematocrit values between females sitting on parasitized and non-parasitized nests. This result suggests that females are not losing much blood to *P. downsi* larvae. Hence, there may be little stimulus for increased corticosterone levels.

It is important to note the short time frame of our study. *P. downsi* can cause nests to fail within a week of the eggs hatching (Koop et al., 2013). We therefore sampled adult female birds for blood relatively quickly (within 4–6 days of the eggs hatching). Since we did not sample females for blood after their nests had failed, we were unable to test whether nest failure itself is a stressor for female finches. Logan and Wingfield (1995) found significant increases in the corticosterone levels of female northern mockingbirds (*Mimus polyglottos*) during incubation of replacement clutches. It is possible that female medium ground finches show an increase in corticosterone levels if and when they re-nest. Further work is needed to determine whether females

experiencing prolonged exposure to *P. downsi* exhibit a corresponding stress response.

It is also possible that significant changes in corticosterone in response to parasitism may be apparent only during "bad" years when birds experience other intense sources of stress. For example, Raouf et al. (2006) showed that adult cliff swallows parasitized by hematophagous swallow bugs have higher baseline corticosterone than non-parasitized birds; however, this was true only for birds nesting in large colonies, which are thought to increase levels of social stress. Cliff swallows in smaller colonies did not show higher levels of corticosterone, perhaps because of reduced competition for food. This result suggests that a combination of stressors can increase corticosterone levels. Our study took place in a year of high rainfall, during which birds were presumably able to find sufficient food and breed readily. Finches do not breed well in years of low rainfall and scarce food supplies (Koop et al., in press). Changes in corticosterone induced by *P. downsi* may be more apparent in dry years, when birds are under greater nutritional stress. It would be interesting to repeat our study in a dry year.

In summary, our results suggest that the impact of *P. downsi* on finch reproductive success is not mediated by a stress response in breeding females. Nestling finches may experience stress responses due to *P. downsi* parasitism; however, we were unable to test this hypothesis because most nestlings died before we could obtain adequate blood samples. Several studies have demonstrated increased levels of corticosterone in nestlings in response to ectoparasites in the nest (Eggert et al., 2010; Kitaysky et al., 2001; Raouf et al., 2006). Breeding adults can leave the nest, temporarily escaping parasites. Nestlings, by contrast, are essentially captive in the nest until they fledge. Thus, nest parasites may be a more intense and persistent stressor to nestlings, exacerbating negative fitness consequences.

## Acknowledgments

We thank Galápagos National Park, the Charles Darwin Foundation, Priscilla Espin Vargas, Miriam Clayton, Lori Neuman-Lee, Leilani Lucus, and Michael Bingham for various forms of assistance. We also thank Fred Adler, Sarah Bush and three anonymous referees for comments that greatly improved the manuscript. The work was supported by NSF grant DEB-0816877 to DHC, the Society of Integrative and Comparative Biology (Grant-in-Aid of Research to SAK), the American Ornithologists' Union (Research Award to SAK), and University of Utah Biology Department (Student Research Award to SAK). All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004).

## References

Boughton, R.K., Atwell, J.W., Schoech, S.J., 2006. An introduced generalist parasite, the sticktight flea (*Echidnophaga gallinacea*), and its pathology in the threatened Florida scrub-jay (*Aphelocoma coerulescens*). J. Parasitol. 92, 941–948.

Breuner, C.W., Hahn, T.P., 2003. Integrating stress physiology, environmental change, and behavior in free-living sparrows. Horm. Behav. 43, 115–123.

Breuner, C.W., Patterson, S.H., Hahn, T.P., 2008. In search of relationships between acute adrenocortical response and fitness. Gen. Comp. Endocrinol. 157, 288–295.

Brown, C.R., Brown, M.B., Raouf, S.A., Smith, L.C., Wingfield, J.C., 2005. Effects of endogenous steroid hormone levels on annual survival in Cliff Swallows. Ecology 86, 1034–1046.

Causton, C.E., Peck, S.B., Sinclair, B.J., Roque-Albelo, L., Hodgson, C.J., Landry, B., 2006. Alien insects: threats and implications for conservation of Galápagos Islands. Ann. Entomol. Soc. Am. 99, 121–143.

Clayton, D.H., Tompkins, D.M., 1994. Ectoparasite virulence is linked to mode of transmission. Proc. R. Soc. 256, 211–217.

Eggert, L.M.F., Jodice, P.G.R., O'Reilly, K.M., 2010. Stress response of brown pelican nestlings to ectoparasite infection. Gen. Comp. Endocrinol. 166, 33–38.

Fessl, B., Couri, M.S., Tebbich, S., 2001. *Philornis downsi* Dodge & Aitken, new to the Galapagos Islands, (Diptera, Muscidae). Studia Dipterologica 8, 317–322.

Fessl, B., Sinclair, B.J., Kleindorfer, S., 2006. The life-cycle of *Philornis downsi* (Diptera: Muscidae) parasitizing Darwin's finches and its impacts on nestling survival. Parasitology 133, 739–747.

Fessl, B., Tebbich, S., 2002. *Philornis downsi* – a recently discovered parasite on the Galápagos archipelago – a threat for Darwin's finches? Ibis 144, 445–451.

French, S.S., DeNardo, D.F., Greives, T.J., Strand, C.R., Demas, G.E., 2010. Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (*Amblyrhynchus cristatus*). Horm. Behav. 58, 792–799.

Grant, P.R., 1999. Ecology and Evolution of Darwin's Finches. Princeton University Press, Princeton, NJ.

Huber, S.K., 2008. Effects of the introduced parasite *Philornis downsi* on nestling growth and mortality in the medium ground finch (*Geospiza fortis*). Biol. Conserv. 141, 601–609.

Huber, S.K., Owen, J.P., Koop, J.A.H., King, M.O., Grant, P.R., Grant, B.R., Clayton, D.H., 2010. Ecoimmunity in Darwin's finches: invasive parasites trigger acquired immunity in the medium ground finch (*Geospiza fortis*). PLoS One 5, e8605.

Johnstone, C.P., Reina, R.D., Lill, A., 2012. Interpreting indices of physiological stress in free-living vertebrates. J. Comp. Physiol. B Biochem. System. Environ. Physiol. 182, 861–879.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 1999. Dynamics of food availability, body condition and physiological stress response in breeding black-legged kittiwakes. Funct. Ecol. 13, 577–584.

Kitaysky, A.S., Wingfield, J.C., Piatt, J.F., 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behav. Ecol. 12, 619–625.

Koop, J.A.H., Le Bohec, C., Clayton, D.H., in press. Dry year does not reduce invasive parasitic fly prevalence or abundance in Darwin's finch nests. Ecol. Evol.

Koop, J.A.H., Huber, S.K., Laverty, S.M., Clayton, D.H., 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PLoS One 6, e19706. http://dx.doi.org/10.1371/journal.pone.0019706.

Koop, J.A.H., Owen, J.P., Knutie, S.A., Aguilar, M.A., Clayton, D.H., 2013. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecol. Evol., http://dx.doi.org/10.1002/ece3.651.

Logan, C.A., Wingfield, J.C., 1995. Hormonal correlates of breeding status, nest construction, and parental care in multiple-brooded northern mockingbirds, *Mimus polyglottos*. Horm. Behav. 29, 12–30.

Love, O.P., Breuner, C.W., Vezina, F., Williams, T.D., 2004. Mediation of a corticosterone-induced reproductive conflict. Horm. Behav. 46, 59–65.

Marshall, A.G., 1981. The Ecology of Ectoparasitic Insects. Academic Press Inc., London.

McEwen, B.S., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine. Horm. Behav. 43, 2–15.

Olaymeni, F.O., 2009. Haematological changes in Guinea fowls (*Numida meleagris galeata*, Pallas) following haemorrhage. Int. J. Poultry Sci. 8, 1093–1095.

Palmer, J., Klugman, K.P., Hattingh, J., 1979. Haematological changes associated with haemorrhage in the pigeon. Comp. Biochem. Phys. A 63, 587–589.

Peig, J., Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118, 1883–1891.

Raouf, S.A., Smith, L.C., Brown, M.B., Wingfield, J.C., Brown, C.R., 2006. Glucocorticoid hormone levels increase with group size and parasite load in cliff swallows. Anim. Behav. 71, 39–48.

Sapolsky, R.M., Romero, M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Schoech, S.J., Mumme, R.L., Wingfield, J.C., 1997. Corticosterone, reproductive status, and body mass in a cooperative breeder, the Florida scrub-jay (*Aphelocoma coerulescens*). Physiol. Zool. 70, 68–73.

Siegel, H.S., 1980. Physiological stress in birds. Bioscience 30, 529–534.

Silverin, B., 1986. Corticosterone-binding proteins and behavioural effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. Gen. Comp. Endocrinol. 64, 67–74.

Sockman, K.W., Schwabl, H., 2001. Plasma corticosterone in nestling American kestrels: effects of age, handling stress, yolk androgens, and body condition. Gen. Comp. Endocrinol. 122, 205–212.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J. Psychosomat. Res. 53, 865–871.

Wingfield, J.C., Kitaysky, A.S., 2002. Endocrine responses to unpredictable environmental events: stress or anti-stress hormones? Integr. Comp. Biol. 42, 600–609.

Wingfield, J.C., Maney, D.L., Breuner, C.W., Jacobs, J.D., Lynn, S., Ramenofsky, M., Richardson, R.D., 1998. Ecological bases of hormone–behavior interactions: the "emergency life history stage". Am. Zool. 38, 191–206.

Wingfield, J.C., Silverin, B., 1986. Effects of corticosterone on territorial behavior of free-living male song sparrows *Melospiza melodia*. Horm. Behav. 20, 405–417.

Wingfield, J.C., Smith, J.P., Farner, D.S., 1982. Endocrine responses of white-crowned sparrows to environmental-stress. Condor 84, 399–409.

# CHAPTER 3

# GALÁPAGOS MOCKINGBIRDS ARE TOLERANT HOSTS OF INTRODUCED PARASITES THAT THREATEN DARWIN'S FINCHES


## Abstract

Introduced parasites threaten native host populations that lack effective defenses. Host species that are relatively unaffected (i.e. tolerant hosts) can allow for the parasite to persist in the environment and increase the threat of infection for vulnerable host populations. Recently, the introduced parasitic nest fly *Philornis downsi* has been implicated in the decline of Darwin's finch populations in the Galápagos Islands. In some years, 100% of finch nests at a given location fail due to *P. downsi*; however, other common host species nesting near Darwin's finches, such as the endemic Galápagos mockingbird (*Mimus parvulus*), appear less affected by *P. downsi*. If *M. parvulus* is unaffected by the nest fly, this species may represent a host that amplifies the threat of *P. downsi* to vulnerable hosts, such as Darwin's finches. The goal of this study was to compare the effects of *P. downsi* on the fitness of mockingbirds and medium ground finches (*Geospiza fortis*). We experimentally manipulated *P. downsi* abundance in the nests of mockingbirds and finches to compare the effects of the parasite on host reproductive fitness. We also examined host immunological and behavioral responses. We found that both species had similar numbers of parasites per gram of host body mass.

*P. downsi* decreased nestling growth and survival of finches only, despite both species losing substantial amounts of blood hemoglobin to the parasite. Among finch nestlings, begging, plasma glucose levels, and parental provisioning did not change in response to *P. downsi*. In contrast, mockingbird nestlings increased energy intake in response to *P. downsi*, with parasitized mockingbird nestlings begging more than nonparasitized nestlings. Greater begging was related to increased parental provisioning, which may have compensated for parasite damage. Our study suggests that finches are negatively affected by *P. downsi* because they do not have an effective behavioral feedback system to compensate for energy lost to the parasite. Furthermore, mockingbirds may indirectly threaten Darwin's finches and other hosts in the Galápagos.

## Introduction

Introduced parasites threaten native host populations that lack effective defenses (Daszak et al. 2000, Keesing et al. 2010). For example, chytrid fungus *Batrachochytrium dendrobatidis* has been implicated in the decline and extinction of many species of amphibians (Daszak et al. 1999, Skerratt et al. 2007). However, not all hosts are vulnerable to introduced parasites; the fitness of some host species is clearly reduced, while the fitness of other host species is relatively unaffected. Such hosts tolerate the effects of the parasite without affecting parasite fitness and can provide a stable resource for the introduced parasite (Schmid-Hempel 2011). In turn, tolerant hosts increase the 'force of infection' for vulnerable host populations, which is the rate of the vulnerable population that the infected hosts are able to contact and infect per unit of time (Anderson and May 1991, Hudson et al. 2002). The Pacific chorus frog (*Pseudacris regilla*) in the Sierra Nevada of California provides such an example. Despite catastrophic declines of

more vulnerable frog species, chorus frogs survive heavy fungal infections (Reeder et al. 2012). These hosts can maintain large numbers of parasites in the environment, even when vulnerable host populations are declining (Nokes 1992) and are an indirect threat to populations of more vulnerable host species (Daszak et al. 2001, McCallum 2012).

Small island populations may be particularly vulnerable to the effects of introduced parasites (Wikelski et al. 2004, Atkinson and Lapointe 2009). A classic example involves the historical introduction of avian malaria parasites and their mosquito vectors to the Hawaiian Islands. This introduction is thought to have been partly responsible for the extinction of 17 endemic honeycreeper species (Atkinson and Lapointe 2009). However, some species of honeycreepers appear to be unaffected by malarial parasites. For example, experiments with captive birds suggest that the amakihi honeycreeper (*Hemignathus virens virens*) is relatively unaffected by the malaria parasite and likely maintains the parasite in the environment (Atkinson et al. 2000). Thus, the amakihi may be a host that amplifies the negative effect of malaria on more vulnerable and declining honeycreeper species (Atkinson and Lapointe 2009).

Direct comparisons of the impact of introduced parasites on different host species in the same community have seldom been carried out under natural conditions. This approach is also difficult because the most rigorous method for assessing the relative effect of parasites on different host species is by experimental manipulation of the parasite (McCallum and Dobson 1995). Comparative experimental studies of the effects of parasites on different host species are important for vulnerable host species with limited population sizes, such as those on islands. Such studies could play a critical role in determining the force of infection within the host-parasite community.

Introduced parasites have colonized the Galápagos Islands of Ecuador in recent decades, threatening endemic species of birds (Wikelski et al. 2004). The most notorious example is the introduced parasitic nest fly *Philornis downsi,* which has been implicated in the decline of critically endangered species of Darwin's finches (O'Connor et al. 2009, Fessl et al. 2010). Adult flies, which are not parasitic, lay their eggs in the nests of finches and other land birds in the Galápagos. Once the fly eggs hatch, the larvae feed on the blood of nestlings and adult females when they sit on the nest. Several studies have shown that *P. downsi* reduces the reproductive success of the medium ground finch (*Geospiza fortis*) and other species of Darwin's finches (reviewed in Koop et al. 2011). In some years, 100% of finch nests at a given location fail to produce fledglings as a direct result of *P. downsi* (Koop et al. 2011, 2013a, O'Connor et al. 2013). Kleindorfer et al. (2014) suggest that in the past decade, finch mortality has increased and age at mortality has decreased due to infestation early in the nestling period by *P. downsi*.

Other common host species nesting near Darwin's finches, such as the endemic Galápagos mockingbird (*Mimus parvulus*), may be less affected by *P. downsi* infestation. If so, then such species could represent hosts that amplify the force of infection of *P. downsi* for vulnerable hosts, such as Darwin's finches. The goal of the current study was to compare the effects of *P. downsi* on the fitness of mockingbirds and medium ground finches at the same time and location. To achieve this goal, we measured the effect of the parasite on nestling mockingbirds and medium ground finches over two field seasons at a single site. During the first season, we compared the effect of *P. downsi* on the size and fledging success of nestling mockingbirds and medium ground finches. We also tested for evidence of nestling immune responses to *P. downsi*. During the second season, we

repeated these comparisons, and we also compared the effect of *P. downsi* on nestling hemoglobin and glucose levels, as well as host behavior.

## Materials and Methods

### Study system

The study was conducted January-April 2012 and 2013 on the island of Santa Cruz in the Galápagos Archipelago. Our field site, El Garrapatero, is 4 x 3 km in the arid coastal zone. Galápagos mockingbirds and medium ground finches are abundant at the site. Mockingbirds build open cup-shaped nests, primarily in giant prickly pear cacti (*Opuntia echios gigantea*) or *Acacia* trees. Mockingbird clutch size ranges from 1-5 eggs, and females incubate the eggs for 12-13 days (Grant and Grant 1979). Nestlings spend an average of 15 days in the nest, and both the adult females and males feed them. Mockingbirds usually lay one clutch of eggs per breeding season; if the nest fails completely, then they may lay a second clutch. Mockingbirds normally do not reuse the same nest.

Finches build dome-shaped nests in giant prickly pear cacti or *Acacia* trees (Grant 1999). Their clutch sizes range from 2-5 eggs and females incubate the eggs for 10-14 days. Nestlings spend an average of 12 days in the nest, and the adult females and males both feed them. In years of favorable weather and food resources, medium ground finches can lay more than one clutch of eggs within a single breeding season; however, like mockingbirds, they do not normally reuse the same nest (Grant 1999).

## Experimental manipulation of parasites

To quantify the effect of *P. downsi* on host fitness, experimental nests were fumigated with a 1% aqueous permethrin solution (Permectrin™ II). Control nests were sham-fumigated with water. Permethrin has been used in previous studies (Fessl et al. 2006, Koop et al. 2013b, 2013a, O'Connor et al. 2013); it is harmless to birds, including newly hatched nestlings. Nests were sprayed soon after the first nestling hatched, then again 4-6 days later. Nest contents (nestlings, unhatched eggs, and liner) were removed during the brief treatment process. The nest contents were returned to the nest after it was dry (<10 minutes). Parents quickly returned to the nest after treatment; no cases of nest abandonment due to treatment were observed for either host species.

## Nestling size and fledging success

In 2012, each nestling was weighed twice: first within 24 hours of hatching, then again at 9-10 days of age. In 2013, each nestling was weighed three times: first within 24 hours of hatching, then again at one-third and two-thirds of the nestling developmental period. Hence, the second weighing occurred when finch nestlings were 4-5 days old, and mockingbird nestlings were 5-6 days old. The third weighing occurred when finch nestlings were 8-9 days old, and mockingbird nestlings were 10-11 days old.

Nestlings were banded with unique color band combinations. Successful fledging was confirmed by identifying birds from their color bands after they had left the nest, as in previous studies (Koop et al. 2011, 2013b, 2013a). After the birds in a nest had fledged or died, the nest was collected and placed in a sealed plastic bag. The number of *P. downsi* in the nests was then quantified, as described below.

## Parasite load

Each nest was carefully dissected within 8 hours of collection and *P. downsi* larvae, pupae, and eclosed pupal cases were counted (Koop et al. 2011, 2013b, 2013a). Parasite density, defined as the number of individual parasites per unit of host (Bush et al. 1997), was determined. For mockingbirds and finches, density was calculated by dividing the number of parasites per nest by the total mass of nestlings in a given nest.

All larvae and pupae were reared to the adult stage to confirm that they were *P. downsi* (Dodge and Aitken 1968). Most larvae were third instars when the nests were collected; these larvae usually pupated within 24 hours. Younger larvae, which require a blood meal, died soon after they were collected from the nest; therefore, they were not reared to adulthood. The length (mm) and width (mm) of pupae were measured with digital calipers. These measurements were used to calculate pupal volume as an estimate of individual parasite size, which is related to lifetime fitness in other Muscid flies (Schmidt and Blume 1973, Moon 1980).

## Nestling hemoglobin

In 2012, blood was sampled from 9-10-day-old nestlings. In 2013, blood was sampled from nestlings when they were at one-third and two-thirds of the nestling period. A small blood sample (< 30 μL) was collected in a microcapillary tube via brachial venipuncture. Using a portion of this blood, hemoglobin concentration was quantified immediately in the field (2013 only). Hemoglobin concentration has been shown to provide accurate estimates of ectoparasite-induced anemia (O'Brien et al. 2001, Carleton 2008). Hemoglobin was measured with a HemoCue® HB 201+ portable

analyzer. Ten microliters of whole blood were placed in a disposable microcuvette, then read on the analyzer; total hemoglobin was measured in g/dL.

The remainder of each blood sample was stored on wet ice in the field. Within 6 hours of collection, the samples were spun at 8000 rpm for 10 minutes in a centrifuge. Plasma and red blood cells were stored separately in 0.5mL vials in a -20°C freezer at the Charles Darwin Research Station. Samples were later frozen at -80°C after being transported in liquid nitrogen to the University of Utah. The samples were subsequently used for immunological and glucose assays; see below.

## Nestling immunology

Enzyme-linked immunosorbent assays (ELISA) were used to detect the presence of *P. downsi*-binding antibodies in the plasma of finches and mockingbirds, using a modification of the protocol in Koop et al. (2013a). Ninety-six-well plates were coated with 100 μL/well of *P. downsi* protein extract (capture antigen) diluted in carbonate coating buffer (0.05M, pH 9.6). Plates were incubated overnight at 4°C, then washed and coated with 200 μL/well of bovine serum albumin (BSA) blocking buffer and incubated for 30 minutes at room temperature on an orbital table. Between each of the following steps, plates were washed five times with a Tris-buffered saline wash solution, loaded as described, and incubated for 1 hour on an orbital table at room temperature. Triplicate wells were loaded with 100 μL/well of individual host plasma (diluted 1:100 in sample buffer). Plates were then loaded with 100 μL/well of Goat-αBird-IgG (diluted 1:50,000)(Antibodies Online). Finally, plates were loaded with 100 μLwell of peroxidase substrate (tetramethylbenzidine, TMB: Bethyl Laboratories) and incubated for

exactly 30 minutes. The reaction was stopped using 100 μL/well of stop solution (Bethyl Laboratories). Optical density (OD) was measured using a spectrophotometer (BioTek, PowerWave HT, 450-nanometer filter).

On each plate, a positive control of pooled plasma from adult female finches was used in triplicate to correct for interplate variation (Koop et al. 2013a). In addition, each plate contained a nonspecific binding (NSB) sample in which capture antigen and detection antibody were added, but plasma was excluded. Finally, each plate included a blank sample in which only the detection antibody was added, but plasma and capture antigen were excluded. NSB absorbance values were subtracted from the mean OD value of each sample.

## Nestling glucose

Plasma glucose was measured using blood samples taken from mockingbird and finch nestlings at around the same time their behavior was quantified; see below. An Endpoint Autokit (Wako, Inc.) was used to measure plasma glucose for mockingbirds and finches with a modified protocol based on Guglielmo et al. (Guglielmo et al. 2013). The kit provided 500 mg/dL and 200 mg/dL standards. From these standards, a standard curve was created ranging from 50-500 mg/dL. Following the manufacturer's protocol, the buffer solution and color reagent were mixed together, then refrigerated at 4°C until used in the assay. Three microliters of sample or standard were run primarily in duplicate, assuming sufficient sample was available, on Nunc® MicroPlate™ 96-well polystyrene plates (Sigma-Alrich). The buffer solution was prewarmed to 37°C, then 300 uL were added to each well. The plate was incubated at 37°C on a microplate incubator shaker (Stat Fax® 2200) for 10 minutes, then shaken for 10 seconds on low

speed. Optical density (OD) was measured using a spectrophotometer (BioTek, PowerWave HT, 505-nanometer filter). Samples were corrected for intraplate variation based on the 500 mg/dL standard. Glucose concentration for each sample was calculated using the slope and intercept of the line from the standard curve ($y = 0.003x + 0.0352$).

## Nestling and adult behavior

Mockingbird behavior was recorded during the 2013 field season; finch behavior was recorded during the 2010 field season at the same location; see Koop et al. (2013a). Host behavior was monitored with battery-powered Sony® video camera systems. Small nest cameras (31 mm in diameter, 36 mm in length) were suspended above nests; 7-meter long cables connected the cameras to small recording devices (PV700 Hi-res DVR, StuntCams) that were hidden near the base of the cactus. Behavior was recorded between 0600 and 1000 in haphazard subsamples of fumigated and sham-fumigated nests.

Nestling behavior was later quantified from the videos at the University of Utah. Nestling begging occurred when one or more nestlings tilted their head back, with the neck extended and the open mouth gape showing (Christe et al. 1996). Begging was quantified as a proportion of total video time. Nestling agitation behavior, defined as shaking, repositioning, or jumping in the nest, was also quantified.

Adult behavior was also quantified from videos. These behaviors included the proportion of time each adult spent at the nest. We were unable to distinguish female and male mockingbirds because mockingbirds are not sexually dimorphic. The following adult behaviors were quantified: brooding nestlings, standing erect in the nest, standing motionless on the rim of the nest, nest sanitation, self-preening, allopreening

nestlings, and provisioning (feeding) nestlings. Brooding was defined as the adult sitting on the nest in direct contact with nestlings. Nest sanitation was defined as the adult contacting or manipulating nest material with its bill. Provisioning of nestlings was defined as the transfer of food from adults to one or more nestlings. Because adults often preen themselves while brooding nestlings, self-preening was analyzed separately from the other behaviors. All other behaviors were analyzed as the proportion of total time that adults engaged in each behavior.

All behaviors were quantified from videos by one of the authors (M.T.) who was blind to nest identity or treatment. Videos were analyzed using the software VLC media player (VideoLAN), except in the case of begging, which was analyzed using CowLog v.2.1 (Hänninen and Pastell 2009). A single day of video from each nest was paired between treatments, based on nestling age and brood size. There was no significant difference in brood size or nestling age between treatments.

Mockingbird behaviors were quantified from a total of 41 hours of video, with an average of 2.5 hours of video for each of the 16 mockingbird nests (eight fumigated, eight sham-fumigated). Mockingbird nestlings in the videos ranged in age from 3 to 6 days, and brood size ranged from 1 to 5 nestlings.

Finch behaviors were quantified from a total of 54 hours of video, with an average of 3 hours of video for each of the 18 finch nests (nine fumigated, nine sham-fumigated) (Koop et al. 2013a). Finch nestlings in the videos ranged in age from 2 to 6 days, and brood size ranged from 1 to 5 nestlings. Adult finch behaviors are also presented separately by sex in Koop et al. (2013a).

### Statistical analyses

Statistical analyses were performed in Prism® v.5.0b (GraphPad Software, Inc.) or SPSS® v.10.0 (SPSS, Inc.).

## Results

### Parasite load

The experimental treatment of nests with permethrin was effective at reducing the abundance of parasites in both mockingbird and finch nests; fumigated nests had few, if any, parasites. Fumigated mockingbird nests had significantly fewer parasites than sham-fumigated nests in both years of the study (Wilcoxon signed rank test: 2012, $W = -120.0$, $P < 0.0001$; 2013, $W = -132.0$, $P < 0.001$). In 2012, fumigated mockingbird nests had a mean ± SE of 0.63 ± 0.44 parasites ($n = 16$), compared to 71.63 ± 17.27 parasites ($n = 16$) in sham-fumigated nests. In 2013, fumigated mockingbird nests had a mean of 0.41 ± 0.31 parasites ($n = 17$), compared to 50.94 ± 11.17 parasites ($n = 17$) in sham-fumigated nests.

Similarly, fumigated finch nests had significantly fewer parasites than sham-fumigated nests in both years of the study (Wilcoxon signed rank test: 2012, $W = -62.0$, $P = 0.007$; Mann-Whitney test: 2013, $U = 20.00$, $P < 0.0001$). In 2012, fumigated finch nests had a mean of 0.25 ± 0.25 parasites ($n = 12$), compared to 26.17 ± 9.09 parasites ($n = 12$) in sham-fumigated nests. In 2013, fumigated finch nests had a mean of zero parasites ($n = 20$), compared to 17.00 ± 3.89 parasites ($n = 17$) in sham-fumigated nests (the 2013 finch nest parasite data were first reported in Knutie et al. (2014).

Parasite density (number of parasites per gram of nestling) did not differ significantly between mockingbirds and finches in either year of the study (Table 3.1;

Mann-Whitney test: 2012, $U = 44.00$, $P = 0.43$; 2013, $U = 78.00$, $P = 0.98$). The size (pupal volume) of parasites in mockingbird and finch nests also did not differ significantly (Table 3.1; Student's t-test: 2012, $t = 0.70$, $df = 20$, $P = 0.49$; 2013, $t = 0.22$, $df = 21$, $P = 0.83$).

## Nestling size

*P. downsi* had no effect on the size of mockingbird nestlings in either year of the study. Nestling mass increased significantly with increasing age in 2012 (two-way ANOVA, age, $F_{1,52} = 1686.00$, $P < 0.0001$; Bonferroni post-hoc tests: $P < 0.05$ for all ages). However, there was no significant effect of fumigation on nestling mass (Fig. 3.1A; treatment, $F_{1,52} = 1.34$, $P = 0.25$). At hatching, mockingbird nestlings from fumigated nests weighed 4.20 ± 0.14g ($n = 15$ nests), compared to 4.10 ± 0.20g ($n = 11$ nests) for nestlings from sham-fumigated nests (Bonferroni post-hoc test: $P > 0.05$). At 9-10 days of age, mockingbird nestlings from fumigated nests weighed 34.32 ± 0.62g ($n = 15$), compared to 32.77 ± 1.15g ($n = 15$) for nestlings from sham-fumigated nests (Fig. 3.1A; $P > 0.05$). There was no significant interaction between age and treatment for nestling mass (age x treatment, $F_{1,52} = 1.00$, $P = 0.32$).

Mockingbird nestling mass also increased significantly with age in 2013 (two-way ANOVA, age, $F_{2,75} = 727.10$, $P < 0.0001$; Bonferroni post-hoc tests: $P < 0.05$ for all ages). There was again no significant effect of fumigation on nestling mass (Fig. 3.1B; treatment, $F_{1,75} = 0.03$, $P = 0.86$). At hatching, mockingbird nestlings from fumigated nests weighed 3.96 ± 0.14g ($n = 14$ nests), compared to 3.93 ± 0.16g ($n = 10$ nests) for nestlings from sham-fumigated nests (Bonferroni post-hoc test: $P > 0.05$). At one-third of the nestling developmental period, mockingbird nestlings from fumigated nests

weighed 18.16 ± 0.84g ($n$ = 15 nests), compared to 18.48 ± 0.80g ($n$ = 15 nests) for nestlings from sham-fumigated nests ($P > 0.05$). At two-thirds of the nestling developmental period, mockingbird nestlings from fumigated nests weighed 35.30 ± 0.62g ($n$ = 14 nests), compared to 34.67 ± 1.32g ($n$ = 13 nests) for nestlings from sham-fumigated nests (Fig. 3.1B; $P > 0.05$). There was no significant interaction between age and treatment for nestling mass (age x treatment, $F_{2,75} = 0.19$, $P = 0.83$).

Finch nestling mass increased significantly with age in 2012 (two-way ANOVA, age, $F_{1,34} = 775.30$, $P < 0.0001$; Bonferroni post-hoc tests: $P < 0.05$ for all ages). There was no significant effect of fumigation on nestling mass, but there was a trend for older nestlings in fumigated nests to be heavier (Fig. 3.1C; treatment, $F_{1,34} = 2.81$, $P = 0.10$). At hatching, finch nestlings from fumigated nests weighed 2.29 ± 0.09g ($n$ = 11 nests), compared to 2.12 ± 0.08g ($n$ = 6 nests) for nestlings from sham-fumigated nests (Bonferroni post-hoc test: $P > 0.05$). The smaller sample size of young nestlings in the sham-fumigated group is due to the discovery of several nests after nestlings were more than 24 hours old. At 9-10 days of age, finch nestlings from fumigated nests weighed 14.97 ± 0.39g ($n$ = 12 nests), compared to 13.68 ± 0.71g ($n$ = 9 nests) for nestlings from sham-fumigated nests (Fig. 3.1C; $P = 0.10$). There was no significant interaction between age and treatment for nestling mass (age x treatment, $F_{1,34} = 1.66$, $P = 0.21$).

Finch nestling mass also increased significantly with age in 2013 (two-way ANOVA, age, $F_{2,83} = 465.20$, $P < 0.0001$; Bonferroni post-hoc tests: $P < 0.05$ for all ages). The larger sample size of finch nests in 2013 revealed a significant effect of fumigation on the mass of older nestlings (Fig. 3.1D; treatment, $F_{1,83} = 5.52$, $P = 0.02$). At hatching, finch nestlings from fumigated nests weighed 2.17 ± 0.08g ($n$ = 13 nests),

compared to 2.22 ± 0.08g ($n$ = 8 nests) for nestlings from sham-fumigated nests (Bonferroni post-hoc test: $P > 0.05$). At one-third of the nestling developmental period, finch nestlings from fumigated nests weighed 7.19 ± 0.22g ($n$ = 18 nests), compared to 6.53 ± 0.27g ($n$ = 17 nests) for nestlings from sham-fumigated nests ($P > 0.05$). At two-thirds of the nestling developmental period, finch nestlings from fumigated nests weighed 13.28 ± 0.31g ($n$ = 18 nests), compared to 11.96 ± 0.54g ($n$ = 15 nests) for nestlings from sham-fumigated nests (Fig. 3.1D; $P < 0.05$). However, there was no significant interaction between age and treatment for nestling mass (age x treatment, $F_{2,83} = 1.94$, $P = 0.15$).

## Fledging success

There was no effect of the experimental manipulation of parasite load on the fledging success of mockingbirds. The number of nestlings that fledged from mockingbird nests did not differ between treatments in either year (Fig. 3.2A). In contrast, there was an effect of the experimental manipulation of parasite load on the fledging success of finches. Sham-fumigated finch nests fledged significantly fewer offspring than fumigated nests in both years of the study (Fig. 3.2B; the 2013 finch data were first published in Knutie et al. (2014).

Comparing fledging success at the level of nests rather than nestlings provided similar results; there was no effect of the experimental manipulation of parasites on the number of mockingbird nests that fledged at least one offspring in either year of the study (Table 3.1; Fisher's exact test: 2012, $P = 1.00$; 2013, $P = 1.00$). However, there was an effect of the experimental manipulation on the number of finch nests that fledged at least

one offspring; the data showed a nonsignificant trend in 2012, and a statistically significant difference in 2013 (Table 3.1; 2012, $P = 0.068$; 2013, $P = 0.002$).

## Nestling hemoglobin

Mockingbird nestling hemoglobin increased significantly with age (Table 3.2; two-way ANOVA: age, $F_{1,55} = 23.37$, $P < 0.0001$; Bonferroni post-hoc test: $P < 0.05$). There was also a significant effect of fumigation on nestling hemoglobin (treatment, $F_{1,55} = 19.28$, $P < 0.0001$). Mockingbird nestlings from fumigated nests had significantly more hemoglobin (30%) than nestlings from sham-fumigated nests two-thirds of the way through the nestling period (Fig. 3.3; Bonferroni post-hoc test: one-third: $P > 0.05$, two-thirds: $P < 0.05$). There was also a significant interaction between age and treatment for hemoglobin (age x treatment, $F_{1,55} = 5.08$, $P = 0.03$).

Finch nestling hemoglobin also increased significantly with age (Table 3.2; two-way ANOVA: age, $F_{1,62} = 30.53$, $P < 0.0001$; Bonferroni post-hoc test: $P < 0.05$). There was also a significant effect of fumigation of nestling hemoglobin (treatment, $F_{1,62} = 8.80$, $P = 0.004$). Finch nestlings from fumigated nests had significantly higher hemoglobin (14%) than nestlings from sham-fumigated nests two-thirds of the way through the nestling period (Fig. 3.3; Bonferroni post-hoc test: one-third: $P > 0.05$, two-thirds: $P < 0.05$). However, there was not a significant interaction between age and treatment for hemoglobin (age x treatment, $F_{1,62} = 0.90$, $P = 0.35$).

## Nestling immunology

Antibody levels were nearly undetectable in mockingbird nestlings in both years. Antibody levels did not differ significantly between 9-10-day-old nestlings in fumigated

and sham-fumigated nests in 2012 (Wilcoxon signed rank test: $W = -44.00$, $P = 0.23$). Mean antibody level in nestlings from fumigated nests was 0.03 ± 0.01 ($n = 15$ nests), compared to 0.04 ± 0.01 ($n = 15$ nests) in nestlings from sham-fumigated nests. In 2013, nestling antibody levels increased significantly between one-third and two-thirds of the nestling developmental period (Table 3.2; two-way ANOVA: age, $F_{1,44} = 29.22$, $P < 0.0001$). However, there was no effect of treatment on antibody level (treatment, $F_{1,44} = 1.02$, $P = 0.32$), nor was there a significant interaction between age and treatment for antibody level (age x treatment, $F_{1,44} = 0.02$, $P = 0.90$).

Antibody levels were also low in finch nestlings in both years. Antibody levels in 9-10-day-old finch nestlings did not differ significantly between treatments in 2012 (Student's t-test: $t = 0.78$, $df = 19$, $P = 0.45$). Mean antibody level in nestlings from fumigated nests was 0.18 ± 0.02 ($n = 12$ nests), compared to 0.23 ± 0.06 ($n = 9$ nests) in nestlings from sham-fumigated nests. In 2013, antibody levels were nearly undetectable in younger finch nestlings (Table 3.2). Antibody levels increased significantly with age (two-way ANOVA: age, $F_{1,55} = 16.67$, $P < 0.0001$); however, there was no effect of treatment on antibody level (treatment, $F_{1,55} = 0.51$, $P = 0.48$), nor a significant interaction between age and treatment for antibody level (age x treatment, $F_{1,55} = 0.03$, $P = 0.86$).

## Nestling glucose

Mockingbird nestlings from fumigated nests had significantly lower glucose levels than nestlings from sham-fumigated nests (Fig. 3.4; Mann-Whitney test: $U = 34.00$, $P = 0.05$). Nestlings from fumigated nests had a mean glucose level of 241.4 ± 4.07 mg/dL ($n = 11$ nests) compared to 265.0 ± 11.15 mg/dL in nestlings from sham-

fumigated nests ($n$ = 12 nests). However, glucose concentration in finch nestlings did not differ significantly between treatments; nestlings from fumigated nests had a mean of 214.2 ± 9.19 mg/dL ($n$ =7 nests) compared to 219.8 ± 9.83 mg/dL in nestlings from sham-fumigated nests ($n$ = 13 nests) (Fig. 3.4; $U$ = 42.00, $P$ = 0.81).

## Nestling and adult behavior

Mockingbird nestlings from sham-fumigated nests spent significantly more time begging than nestlings from fumigated nests (Table 3.3; Fig. 3.5A). In contrast, mockingbird nestling agitation did not differ significantly between fumigated and sham-fumigated nests (Table 3.3).

The amount of time adult mockingbirds spent at fumigated and sham-fumigated nests did not differ significantly (Table 3.3). Adults spent less than 0.01% of their time at the nest self-preening, which was not exclusive of other behaviors, and there was no significant effect of treatment on self-preening ($W$ = -3.00, $P$ = 0.81).

Adults differed significantly in the time they devoted to mutually exclusive behaviors at fumigated versus sham-fumigated nests (Chi-square test: $\chi^2$ = 18.90, $df$ = 5, $P$ < 0.001). The largest difference was the amount of time adult mockingbirds spent brooding, with adults at fumigated nests spenting significantly more time brooding than adults at sham-fumigated nests (Table 3.3). When mockingbirds from sham-fumigated nests were not brooding, but were still at the nest, they were either standing erect in the nest, or they were standing erect on the rim of the nest. Adults on the rim of the nest were sometimes also probing nest material (nest sanitation), allopreening nestlings, or provisioning nestlings. The amount of time adults spent standing erect in the nest did not differ significantly between fumigated and sham-fumigated nests (Table 3.3). Similarly,

the amount of time adults spent standing erect (and motionless) on the rim of the nest did not differ significantly between fumigated and sham-fumigated nests (Table 3.3).

Adult mockingbirds spent very little time engaged in nest sanitation, and there was not a significant effect of treatment on sanitation (Table 3.3). When adult mockingbirds from sham-fumigated nests were not brooding but still at the nest, they spent most of this time allopreening nestlings while standing on the rim of the nest, although the difference between treatments was not significant (Table 3.3). Adults from fumigated nests spent significantly less time provisioning nestlings, compared to adults from sham-fumigated nests (Table 3.3; Fig. 3.5A). Furthermore, the amount of time parents provisioned nestlings was positively correlated with the amount of time nestlings spent begging (Spearman rank correlation: $r_s = 0.52$, $P = 0.04$).

In the case of finches, the amount of time that nestlings begged did not differ significantly between fumigated and sham-fumigated nests (Table 3.3; Fig. 3.5B). The amount of time adult finches spent at fumigated and sham-fumigated nests did not differ significantly (Table 3.3). The amount of time parents spent provisioning nestlings was correlated with nestling begging time (Spearman rank correlation: $r_s = 0.81$, $P < 0.0001$). However, adult finches did not differ significantly in the amount of time they spent provisioning nestlings at fumigated nests, compared to sham-fumigated nests (Table 3.3; Fig. 3.5B).

## Discussion

The effect of *P. downsi* varied considerably between finches and mockingbirds. As shown in previous studies (Koop et al. 2011, 2013a, O'Connor et al. 2013), *P. downsi* reduced the survival of Darwin's finch nestlings; the parasite also had an effect on finch

body mass. Despite similar parasite densities in finches and mockingbirds, *P. downsi* had no effect on mockingbird nestling survival or body mass. The parasite reduced hemoglobin in both mockingbirds and finches. Mockingbird nestlings from sham-fumigated nests begging significantly more than nestlings from fumigated nests. Greater begging was correlated with increased parental provisioning. Interestingly, mockingbirds increased their energy intake in response to *P. downsi*. In contrast, finch nestling begging and parental provisioning did not change in response to *P. downsi*, nor was there a difference in the plasma glucose levels of nestlings in fumigated and sham-fumigated nests.

Our results suggest that mockingbirds are relatively unaffected by *P. downsi* and may increase the force of infection for Darwin's finches. Previous studies have identified such hosts using observational or correlational data, rather than by directly comparing the effect of parasites on different host species (Atkinson et al. 2000, Haydon et al. 2002). More recent field studies have focused primarily on introduced tolerant hosts (Laurenson et al. 2003). However, few studies have described the relative importance of native host species as tolerant hosts of an introduced parasite (Atkinson et al. 2000). In our system, *P. downsi* appears to maintain a large population size. This may be partly because they can parasitize tolerant Galápagos mockingbirds, regardless of potentially declining Darwin's finch host populations (Appendix B). Mockingbirds are therefore an indirect threat to Darwin's finches, such as the critically endangered mangrove finch, whose population is declining, in part, due to *P. downsi* (Fessl et al. 2010). The dramatic difference in the effect of *P. downsi* on mockingbirds and finches then poses the question,

why are some host species relatively unaffected by a particular parasite, while other host species suffer mortality?

Neither mockingbird nor finch nestlings produced a significant antibody-mediated immune response to *P. downsi* in our study. In fact, antibody levels in nestlings were nearly undetectable, compared to adults (Koop et al. 2013a). Captive house sparrows (*Passer domesticus*) are able to produce an independent antibody-mediated immune response as early as 3 days old when challenged with a nonspecific antigen (King et al. 2010); it is possible that finch and mockingbird nestlings are incapable of mounting a robust immune response to *P. downsi* or our assay was not sensitive to detect low concentrations of antibodies. Antibody levels increased with nestling age, but the response did not differ significantly between treatments. This may be because the antibodies detected were not highly specific to *P. downsi*. Instead, the antibodies may have been a response to other biting insects, such as mosquitoes, which have antigens in their saliva that induce similar responses to those induced by *P. downsi* (e.g. IgG) in their host (Peng et al. 1996). Our results suggest that a nestling immune response does not ameliorate the effect of *P. downsi* on mockingbirds.

Mockingbird parents from sham-fumigated nests brooded their nestlings less than parents from fumigated nests. These adults were still present at the nest, but they may have been trying to avoid the parasites themselves by standing on the rim of the nest. Koop et al. (2013a) found that finches also brood their nestlings less and stand in the nests more in sham-fumigated nests, probably to avoid parasites. However, unlike finches, mockingbird parents were also allopreening their nestlings. It is unclear from our video analyses whether parents are successful in removing or injuring *P. downsi*, as

shown in studies of some other systems (reviewed in Clayton et al. 2010), but this behavior could serve as an effective defense against *P. downsi*. Further tests are needed to determine the extent to which mockingbirds can reduce *P. downsi* in nests through allopreening.

Mockingbirds appear to tolerate effects of *P. downsi* by increasing parental provisioning of nestlings to compensate for energy lost to the parasite. In other systems, parasitic flies can increase host metabolic rate, which depletes host energy resources (Careau et al. 2010). Several studies of other systems have shown that parents from sham-fumigated nests feed their nestlings more than parents from fumigated nests, which increases nestling survival (Tripet and Richner 1997, Hurtrez-Bousses et al. 1998, Tripet et al. 2002). We also found that increased begging by mockingbird nestlings from sham-fumigated nests was met with more food by the parents, which likely increased nestling survival. Thus, one apparent mechanism for dealing with some parasite species is an effective behavioral feedback system between nestlings and parents.

Why are finch nestlings not begging more when parasitized by *P. downsi*? The answer may be that smaller bodied hosts are unable to tolerate the effect of the parasite (finch nestlings are half the size of mockingbird nestlings). Smaller birds require more energy per gram of body mass because they have a higher body surface area-to-volume ratio compared to larger birds (reviewed in Schmidt-Nielsen 1984). Thus, smaller bird species tend to beg more often than larger bird species (Price and Ydenberg 1995, Christe et al. 1996, Leech and Leonard 1996, Kitaysky et al. 2001, Saino et al. 2001, Simon et al. 2005) and they may be fed more often by their parents (Christe et al. 1996). Indeed, finch nestlings from fumigated nests spent more than twice as much time begging as

mockingbirds. Because begging in small birds is more energetically costly (per gram) than begging in larger birds (Jurisevic et al. 1999), finch nestlings may experience a ceiling effect, in which they are energetically incapable of increasing the rate of begging when parasitized.

Other small species are able to increase begging in response to native parasitic flies (Christe et al. 1996), which suggests that finches may be able to evolve this behavioral feedback system. A future study could test whether parasitized finch nestlings would receive more food if they begged more, by artificially increasing begging calls in nests with *P. downsi*. Prerecorded begging audio in the nest may increase food delivery rates by parents (Bengtsson and Rydén 1983, Ottosson et al. 1997). In turn, increased parental provisioning might increase finch nestling survival.

Our study is one of the first to show differential tolerance to an introduced parasite in a natural community. Another important result from our study is that mockingbirds have tolerance mechanisms for dealing with *P. downsi.* Mockingbirds can alleviate the effect of the parasite without reducing parasite abundance. Only recently has the idea of animal host tolerance to parasitism become more widely recognized as an important defense strategy for explaining host-parasite dynamics (Read et al. 2008, Råberg et al. 2009, Baucom and de Roode 2011, Medzhitov et al. 2012, Sorci 2013). Future studies could focus on defense strategies in host species related to Darwin's finches and Galápagos mockingbirds in the native range of *P. downsi*. These tests could help to determine whether: 1) mockingbirds also are unaffected in the native range of *P. downsi,* and 2) Darwin's finches have the potential to evolve effective tolerance defense mechanisms against *P. downsi* in the near future.

## Acknowledgments

This manuscript will be submitted for publication with Jeb P. Owen, Sabrina M. McNew, Andrew W. Bartlow, Elena Arriero, Jordan M. Herman, Emily DiBlasi, Michael Thompson, Jennifer A.H. Koop, and Dale H. Clayton. We thank Daniela Vargas, Michael Skinner, Oliver Tisalema, and Joost Raeymaekers for assistance in the field, and Christopher Guglielmo for advice on the glucose assays. We thank the Galápagos National Park and Charles Darwin Foundation for assistance in working in the Galápagos. We thank Franz Goller, Wayne Potts, and Denise Dearing, Luis Ortiz Catedral, Francesca Cunningham, Paquita Hoeck, and Sarah Bush for helpful discussions on the study. The work was supported by NSF grant DEB-0816877 to DHC and University of Utah Global Change and Sustainability research grant and a Frank Chapman student research grant to SAK. SAK was supported by a University of Utah Graduate Research Fellowship. AWB and SMM were supported by NSF Graduate Research Fellowships.

## References

Anderson, R. M., and R. M. May. 1991. Infectious Diseases of Humans: Dynamics and Control. Page 768. Oxford University Press, Oxford.

Atkinson, C. T., R. J. Dusek, K. L. Woods, and W. M. Iko. 2000. Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. Journal of Wildlife Diseases 36:197–204.

Atkinson, C. T., and D. A. Lapointe. 2009. Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. Journal of Avian Medicine and Surgery 23:53–63.

Baucom, R. S., and J. C. de Roode. 2011. Ecological immunology and tolerance in plants and animals. Functional Ecology 25:18–28.

Bengtsson, H., and O. Rydén. 1983. Parental feeding rate in relation to begging behavior in asynchronously hatched broods of the great tit *Parus major*: An experimental study. Behavioral Ecology and Sociobiology 12:243–251.

Bush, A. O., K. D. Lafferty, J. M. Lotz, and A. W. Shostak. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology 83:575–583.

Careau, V., D. W. Thomas, and M. M. Humphries. 2010. Energetic cost of bot fly parasitism in free-ranging eastern chipmunks. Oecologia 162:303–312.

Carleton, R. E. 2008. Ectoparasites affect hemoglobin and percentages of immature erythrocytes but not hematocrit in nestling Eastern Bluebirds. The Wilson Journal of Ornithology 120:565–568.

Christe, P., H. Richner, and A. Oppliger. 1996. Begging, food provisioning, and nestling competition in great tit broods infested with ectoparasites. Behavioral Ecology 7:127–131.

Clayton, D. H., J. A. H. Koop, C. W. Harbison, B. R. Moyer, and S. E. Bush. 2010. How Birds Combat Ectoparasites. The Open Ornithology Journal 3:41–71.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735–748.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443–449.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica 78:103–116.

Dodge, H. R., and T. H. G. Aitken. 1968. *Philornis* Flies from Trinidad (Diptera: Muscidae). Journal of the Kansas Entomological Society 41:134–154.

Fessl, B., S. Kleindorfer, and S. Tebbich. 2006. An experimental study on the effects of an introduced parasite in Darwin's finches. Biological Conservation 127:55–61.

Fessl, B., G. H. Young, R. P. Young, J. Rodríguez-Matamoros, M. Dvorak, S. Tebbich, and J. E. Fa. 2010. How to save the rarest Darwin's finch from extinction: the mangrove finch on Isabela Island. Philosophical Transactions of the Royal Society of London B 365:1019–1030.

Grant, P. 1999. Ecology and Evolution of Darwin's finches. Princeton University Press, Princeton, New Jersey.

Grant, P. R., and N. Grant. 1979. Breeding and feeding of Galápagos Mockingbirds *Nesomimus parvulus*. Auk 96:723–736.

Guglielmo, C. G., P. D. O. Hara, and T. D. Williams. 2013. Extrinsic and intrinsic sources of variation in plasma lipid metabolites of free-living western sandpipers (*Calidris mauri*). Auk 119:437–445.

Hänninen, L., and M. Pastell. 2009. CowLog: open-source software for coding behaviors from digital video. Behavior Research Methods 41:472–476.

Haydon, D. T., S. Cleaveland, L. H. Taylor, and M. K. Laurenson. 2002. Identifying reservoirs of infection: a conceptual and practical challenge. Emerging Infectious Diseases 8:1468–1473.

Hudson, P. J., A. P. Rizzoli, B. T. Grenfell, J. A. P. Heesterbeek, and A. P. Dobson. 2002. The Ecology of Wildlife Diseases. Page 197. Oxford University Press, Oxford.

Hurtrez-Bousses, S., J. Blondel, P. Perret, J. Fabreguettes, and F. R. Renaud. 1998. Chick parasitism by blowflies affects feeding rates in a Mediterranean population of blue tits. Ecology Letters 1:17–20.

Jurisevic, M. A., K. J. Sanderson, and R. V Baudinette. 1999. Metabolic rates associated with distress and begging calls in birds. Physiological and Biochemical Zoology 72:38–43.

Keesing, F., L. K. Belden, P. Daszak, A. Dobson, C. D. Harvell, R. D. Holt, P. Hudson, A. Jolles, K. E. Jones, C. E. Mitchell, S. S. Myers, T. Bogich, and R. S. Ostfeld. 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468:647–652.

King, M. O., J. P. Owen, and H. G. Schwabl. 2010. Are maternal antibodies really that important? Patterns in the immunologic development of altricial passerine house sparrows (Passer domesticus). PloS One 5:e9639.

Kitaysky, A. S., J. C. Wingfield, and J. F. Piatt. 2001. Corticosterone facilitates begging and affects resource allocation in the black-legged kittiwake. Behavioral Ecology 12:619–625.

Kleindorfer, S., K. J. Peters, G. Custance, R. Y. Dudaniec, and J. A. O'Connor. 2014. Changes in *Philornis* infestation behavior threaten Darwin's finch survival. Current Zoology 60:542–550.

Knutie, S. A., S. M. McNew, A. W. Bartlow, D. A. Vargas, and D. H. Clayton. 2014. Darwin's finches combat introduced nest parasites with fumigated cotton. Current Biology 24:R355–R356.

Koop, J. A. H., J. P. Owen, S. A. Knutie, M. A. Aguilar, and D. H. Clayton. 2013a. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecology and Evolution 3:2514–2523.

Koop, J. A., S. K. Huber, S. M. Laverty, and D. H. Clayton. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PloS One 6:e19706.

Koop, J. A., C. LeBohec, and D. H. Clayton. 2013b. Dry year does not reduce invasive parasitic fly prevalence or abundance in Darwin's finch nests. Reports in Parasitology 3:11–17.

Laurenson, M. K., R. A. Norman, L. Gilbert, H. W. Reid, and P. J. Hudson. 2003. Identifying disease reservoirs in complex systems: mountain hares as reservoirs of ticks and louping-ill virus, pathogens of red grouse. Journal of Animal Ecology 72:177–185.

Leech, S. M., and M. L. Leonard. 1996. Is there an energetic cost to begging in nestling tree swallows (*Tachycineta bicolor*)? Proceedings of the Royal Society of London B: Biological Sciences 263:983–987.

McCallum, H. 2012. Disease and the dynamics of extinction. Philosophical Transactions of the Royal Society of London B: Biological Sciences 367:2828–2839.

McCallum, H., and A. Dobson. 1995. Detecting disease and parasite threats to endangered species and ecosystems. Trends in Ecology and Evolution 10:190–194.

Medzhitov, R., D. Schneider, and M. Soares. 2012. Disease tolerance as a defense strategy. Science 335:936–941.

Moon, R. 1980. Effects of larval competition on face fly. Environmental Entomology 9:325–330.

Nokes, D. J. 1992. Microparasites: viruses and bacteria. Pages 349–374 *in* M. J. Crawley, editor. Natural enemies: the population biology of predators, parasites and diseases. Blackwell Scientific, Boston.

O'Brien, E., B. Morrison, and L. Scott Jonhson. 2001. Assessing the effects of haematophagous ectoparasites on the health of nestling birds: haematocrit vs haemoglobin levels in house wrens parasitized by blow fly. Journal of Avian Biology 32:73–76.

O'Connor, J. A., J. Robertson, and S. Kleindorfer. 2013. Darwin's finch begging intensity does not honestly signal need in parasitised nest. Ethology 119:1–10.

O'Connor, J. A., F. J. Sulloway, J. Robertson, and S. Kleindorfer. 2009. *Philornis downsi* parasitism is the primary cause of nestling mortality in the critically endangered Darwin's medium tree finch (*Camarhynchus pauper*). Biodiversity and Conservation 19:853–866.

Ottosson, U., J. Bäckman, and H. G. Smith. 1997. Begging affects parental effort in the pied flycatcher, *Ficedula hypoleuca*. Behavioral Ecology and Sociobiology 41:381–384.

Peng, Z., M. Yang, and F. E. R. Simons. 1996. Immunologic mechanisms in mosquito allergy: correlation of skin reactions with specific IgE and IgG antibodies and lymphocyte proliferation response to mosquito antigens. Annals of Allergy, Asthma and Immunology 77:238–244.

Price, K., and R. Ydenberg. 1995. Begging and provisioning in broods of asynchronously-hatched yellow-headed blackbird nestlings. Behavioral Ecology and Sociobiology 37:201–208.

Råberg, L., A. L. Graham, and A. F. Read. 2009. Decomposing health: tolerance and resistance to parasites in animals. Philosophical Transactions of the Royal Society of London B: Biological Sciences 364:37–49.

Read, A. F., A. L. Graham, and L. Råberg. 2008. Animal defenses against infectious agents: is damage control more important than pathogen control. PLoS Biology 6:e4.

Reeder, N. M. M., A. P. Pessier, and V. T. Vredenburg. 2012. A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PloS One 7:e33567.

Saino, N., M. Incagli, R. Martinelli, R. Ambrosini, and A. P. Moller. 2001. Immunity, growth and begging behaviour of nestling Barn Swallows *Hirundo rustica* in relation to hatching order. Journal of Avian Biology 32:263–270.

Schmid-Hempel, P. 2011. Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics. Page 544. Oxford University Press, Oxford.

Schmidt, C. D., and R. R. Blume. 1973. Laboratory-reared horn flies: relationships between width of head and weight of pupa in both sexes and between these measurements and number of ovarioles in females. Annals of the Entomological Society of America 66:1307–1308.

Schmidt-Nielsen, K. 1984. Scaling: Why is Animal Size so Important? Page 256. Cambridge University Press, Cambridge.

Simon, A., D. W. Thomas, J. R. Speakman, J. Blondel, P. Perret, and M. M. Lambrechts. 2005. Impact of ectoparasitic blowfly larvae (*Protocalliphora* spp.) on the behavior and energetics of nestling Blue Tits. Journal of Field Ornithology 76:402–410.

Skerratt, L. F., L. Berger, R. Speare, S. Cashins, K. R. McDonald, A. D. Phillott, H. B. Hines, and N. Kenyon. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. EcoHealth 4:125–134.

Sorci, G. 2013. Immunity, resistance and tolerance in bird-parasite interactions. Parasite Immunology 35:350–361.

Tripet, F., M. Glaser, and H. Richner. 2002. Behavioural responses to ectoparasites: time-budget adjustments and what matters to blue tits *Parus caeruleus* infested by fleas. Ibis 144:461–469.

Tripet, F., and H. Richner. 1997. Host responses to ectoparasites: food compensation by parent blue tits. Oikos 78:557–561.

Wikelski, M., J. Foufopoulos, H. Vargas, and H. Snell. 2004. Galápagos birds and diseases: invasive pathogens as threats for island species. Ecology and Society 9:online: http://www.ecologyandsociety.org/vol9/iss1.

Table 3.1. Comparison of *P. downsi* number and size, and host fledging success in mockingbirds and finches in fumigated (F) and sham-fumigated (SF) nests. See text for stats.

| | **Galápagos mockingbird** | | | | **Medium ground finch** | | | |
|---|---|---|---|---|---|---|---|---|
| | **2012** | | **2013** | | **2012** | | **2013** | |
| | **F** | **SF** | **F** | **SF** | **F** | **SF** | **F** | **SF** |
| Mean±SE parasite density (# of nests) | — | 1.00±0.29 (14) | — | 1.06±0.47 (13) | — | 1.49±0.52 (8) | — | 1.06±0.42 (12) |
| Mean±SE pupal volume, $mm^3$ (# of nests) | — | 115.20±6.57 (13) | — | 120.10±8.52 (14) | — | 108.30±6.93 (9) | — | 117.50±5.70 (9) |
| Fledglings, % (# of nestlings) | 76.5% (51) | 77.8% (54) | 70.0% (47) | 66.7% (54) | 86.0% (43) | 34.2% (38) | 83.3% (60) | 53.7% (54) |
| Nests with at least one fledgling, % (# of nests) | 87.5% (16) | 87.5% (16) | 76.5% (17) | 76.5% (17) | 91.7% (12) | 50.0% (12) | 95.0% (20) | 64.7% (17) |

Table 3.2. Comparison of nestling parameters for mockingbirds and finches in fumigated and sham-fumigated nests. Data were collected at 1/3 and 2/3 of the nestling development period (NP) in 2013. Numbers represent the grand mean (±SE) across all nests. Antibody levels are measured by optical density (OD). See text for stats.

| | **Galápagos mockingbird** | | | | **Medium ground finch** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Fumigated** | | **Sham-fumigated** | | **Fumigated** | | **Sham-fumigated** | |
| | **1/3 NP** | **2/3 NP** | **1/3 NP** | **2/3 NP** | **1/3 NP** | **2/3 NP** | **1/3 NP** | **2/3 NP** |
| Hemoglobin, g/dL (# of nests) | 7.30±0.24 (14) | 10.50±0.31 (14) | 6.33±0.39 (17) | 7.50±0.72 (14) | 8.18±0.20 (19) | 10.53±0.19 (18) | 7.45±0.49 (16) | 9.11±0.57 (13) |
| Antibody levels, OD (# of nests) | 0.01±0.00 (13) | 0.04±0.01 (12) | 0.02±0.01 (12) | 0.05±0.01 (11) | 0.03±0.01 (15) | 0.06±0.01 (19) | 0.02±0.00 (14) | 0.05±0.01 (11) |

Table 3.3. Comparison of nestling and adult behaviors for mockingbirds and finches in fumigated and sham-fumigated nests. For mockingbirds, each treatment contained eight nests; for finches, each treatment contained nine nests. Wilcoxon signed rank tests were used to compare treatments for each behavior.

| | **Fumigated** | **Sham-fumigated** | ***W* statistic** | ***P*-value** |
|---|---|---|---|---|
| **Galápagos mockingbird** | | | | |
| Nestlings | | | | |
| Begging | 3.12 ± 0.74% | 5.78 ± 0.98% | -32.00 | 0.02 |
| Agitation | 10.38 ± 2.16% | 12.86 ± 3.07% | -6.00 | 0.74 |
| Adults | | | | |
| Attendance at nest | 54.59 ± 5.00% | 50.45 ± 6.78% | 10.00 | 0.55 |
| Brooding | 70.35 ± 5.05% | 41.12 ± 8.11% | 32.00 | 0.02 |
| Standing erect in nest | 2.27 ± 0.57% | 9.03 ± 7.46% | 8.00 | 0.64 |
| Standing on rim | 7.77 ± 1.85% | 11.87 ± 2.65% | -18.00 | 0.25 |
| Nest sanitation | 0.92 ± 0.43% | 1.24 ± 0.32% | -16.00 | 0.31 |
| Allopreening | 15.18 ± 3.85% | 30.77 ± 7.36% | -24.00 | 0.11 |
| Provisioning nestlings | 3.50 ± 0.56% | 5.98 ± 1.04% | -32.00 | 0.02 |
| **Medium ground finch** | | | | |
| Nestlings | | | | |
| Begging | 6.85 ± 0.90% | 5.53 ± 0.86% | 25.00 | 0.16 |
| Adults | | | | |
| Attendance at nest | 47.29 ± 6.10% | 57.72 ± 9.14% | -23.00 | 0.20 |
| Provisioning nestlings | 11.05 ± 2.19% | 10.45 ± 4.90% | 27.00 | 0.13 |

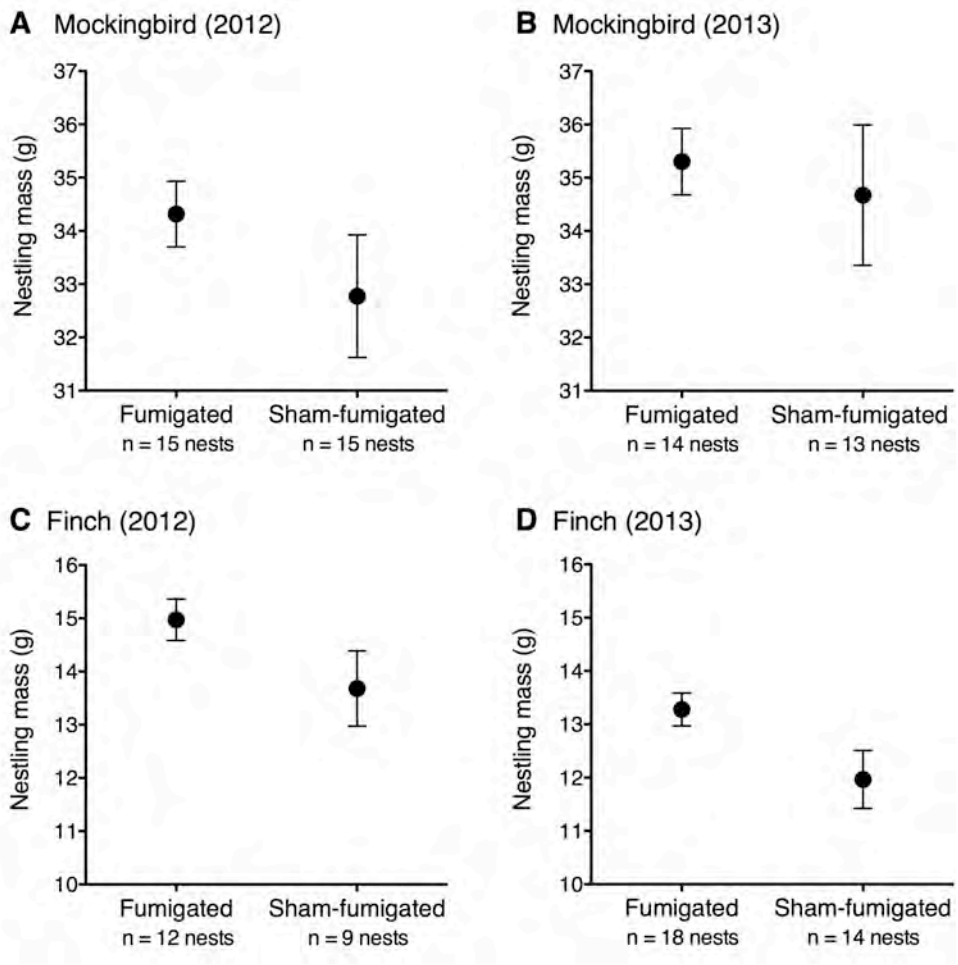


Fig. 3.1. Comparison of the mean (± SE) mass of nestlings in fumigated versus sham-fumigated nests for mockingbirds (A, B) and finches (C, D). Mockingbird nestlings in fumigated and sham fumigated nests did not differ significantly in mass at 9-10 days of age (A, 2012) or 10-11 days of age (B, 2013). Finch nestlings in fumigated nests tended to be heavier than nestlings in sham-fumigated nests at 9-10 days of age (C, 2012); nestlings in fumigated nests were significantly heavier than nestlings in sham-fumigated nests at 8-9 days of age (D, 2013). The lower sample size of sham-fumigated finch nests in 2012 (C) was caused by higher mortality of nestlings prior to weighing. See text for further details.

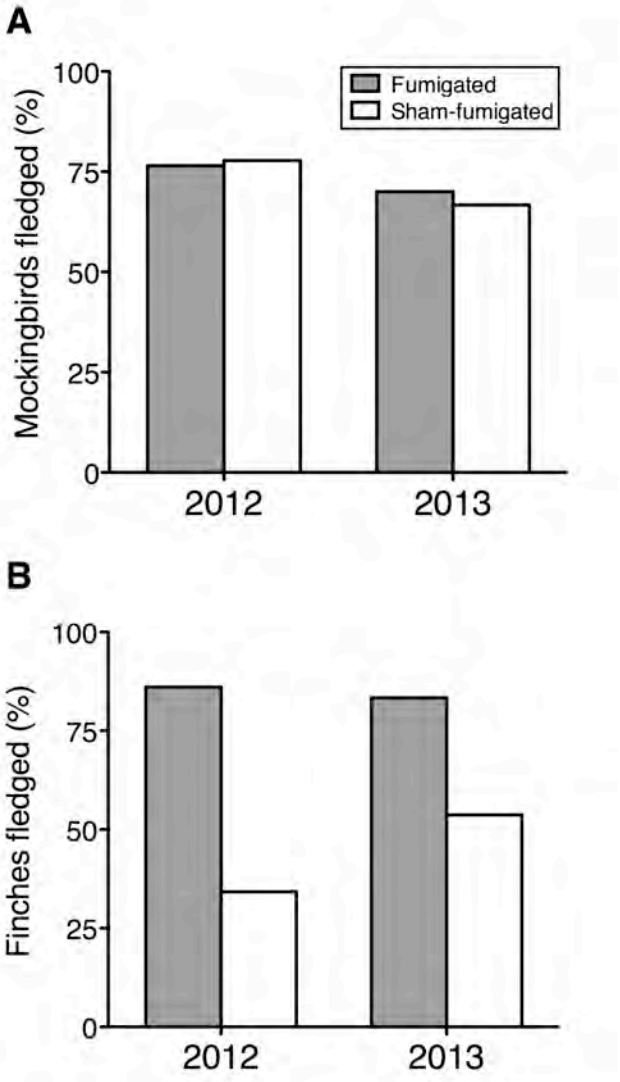


Fig 3.2. Fledging success of mockingbird (A) and finch (B) nestlings in fumigated and sham-fumigated nests. For mockingbirds, fledging success did not differ between treatments in 2012 (Fisher's exact test, $P = 1.00$) or 2013 ($P = 0.83$). For finches, sham-fumigated nests fledged significantly fewer offspring than fumigated nests in both 2012 ($P < 0.0001$) and 2013 ($P = 0.001$).

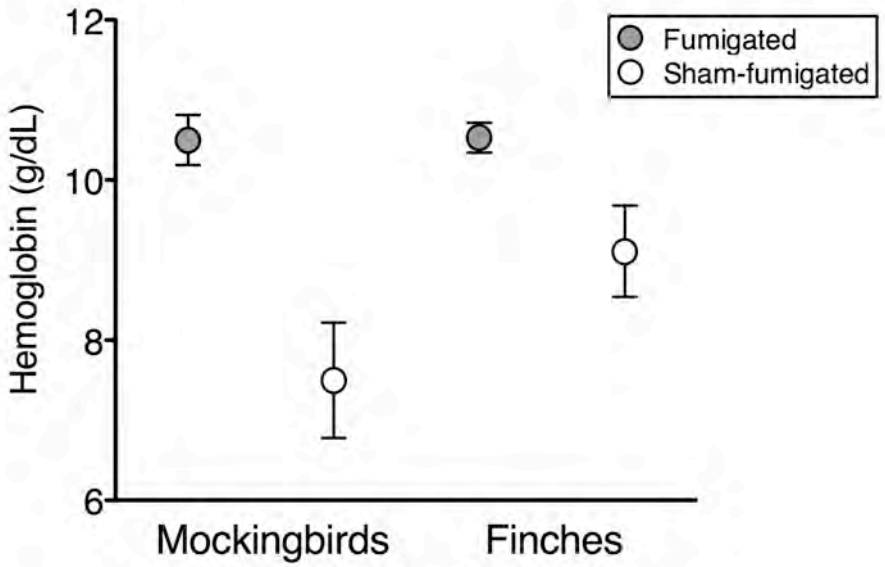


Fig 3.3. Mean (± SE) hemoglobin in nestlings from fumigated and sham-fumigated nests. Nestlings from fumigated nests had significantly higher hemoglobin levels than nestlings from sham-fumigated nests in both species of birds. See text for further details.

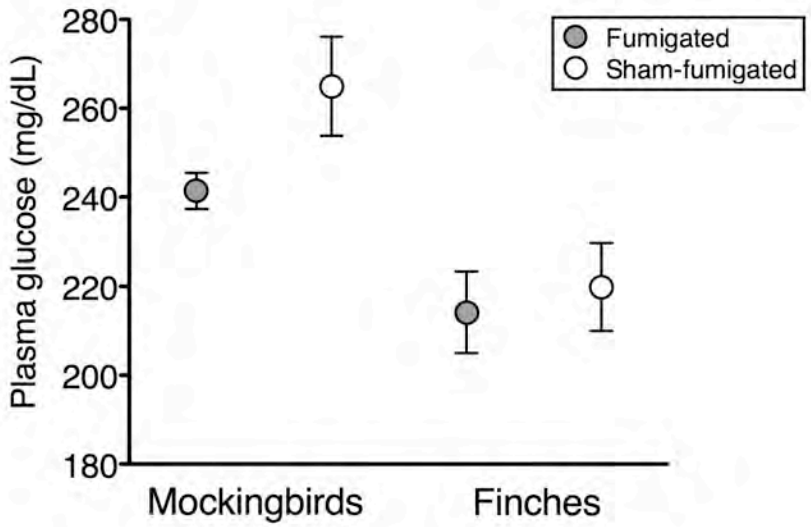


Fig. 3.4. Mean (± SE) plasma glucose levels in mockingbird and finch nestlings from fumigated and sham-fumigated nests. Mockingbird nestlings from sham-fumigated nests had higher glucose levels than nestlings from fumigated nests. In contrast, glucose levels did not differ significantly between treatments for finches. See text for further details.

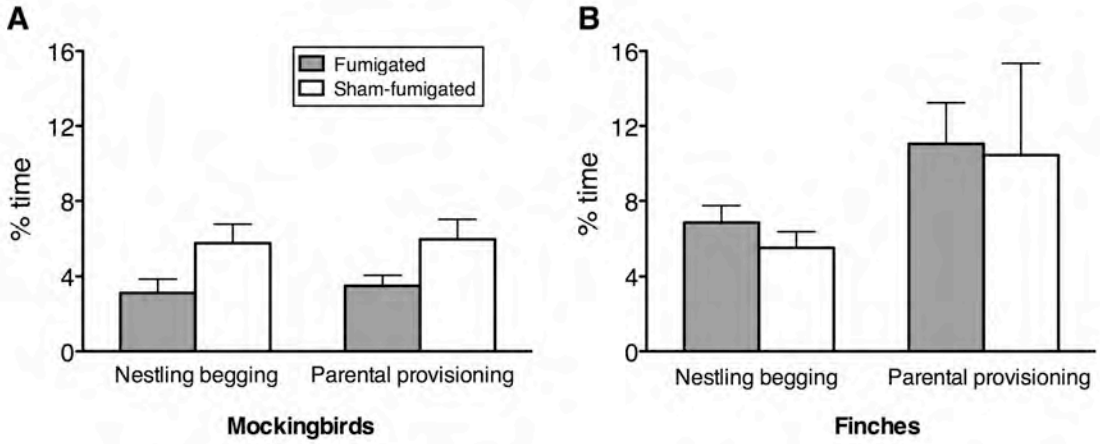


Fig. 3.5. Nestling and parental behavior (mean +SE) in fumigated and sham-fumigated nests for A) mockingbirds and B) finches. The amount of time allocated to nestling begging and parental provisioning was significantly higher in sham-fumigated mockingbird nests compared to fumigated nests. In contrast, the amount of time spent on these behaviors did not differ significantly between treatments in finches. See text for further details.

# CHAPTER 4

# THE EFFECT OF AN INTRODUCED PARASITE ON GENE EXPRESSION IN TWO SPECIES OF GALÁPAGOS BIRDS


## Abstract

Introduced parasites can severely affect the fitness of their naïve hosts. However, the effect of such parasites on host gene expression is poorly understood. The introduced parasitic nest fly *Philornis downsi* has been implicated in the decline of endangered species of Darwin's finches. However, endemic Galápagos mockingbirds (*Mimus parvulus*) are relatively unaffected by the parasite. We explored the potential genetic mechanisms that may promote differences in the effect of the parasite on hosts. We compared gene expression from erythrocytes of parasitized and nonparasitized nestling medium ground finches (*Geospiza fortis*) and mockingbirds. We found that a significant number of genes were expressed differently in mockingbirds and finches when exposed to the parasite. More genes were expressed differently and more KEGG pathways were significantly affected in parasitized mockingbirds, compared to parasitized finches. Few differentially expressed genes in response to parasitism were the same in both species, suggesting that the genetic basis of the effect of the parasite is host species specific. Parasitized hosts did not demonstrate differential expression of genes related to immune responses. Instead, *P. downsi* affected a significant number of host genes related to other

physiological processes, such as metabolism. Notably, parasitized finches had significantly more expression in genes related to DNA repair, compared to mockingbirds; this suggests that more cellular and DNA damage occurred in finches, with downstream effects on development and survival. Our study suggests that the introduced parasitic nest fly has a significant, but species-specific, effect on gene expression of nestling of different host species in the Galápagos.

## Introduction

Organisms face increasing pressure to adapt to changing environments or they may suffer population declines or even local extinction (Jetz et al. 2007, Keesing et al. 2010, Pimm et al. 2014). Introduced parasite species are thought to be one of the leading causes of native vertebrate extinctions (Vitousek et al. 1997, Daszak et al. 1999, 2000, Keesing et al. 2010). Host populations that are unable to evolve effective defenses against introduced parasites are at risk (Atkinson and Lapointe 2009). Although host defenses against such parasites have been studied, the underlying genetic basis for host-parasite interactions are relatively unexplored (Rosenblum et al. 2009). Changes in host gene expression in response to parasites can provide insight into the biological mechanisms and pathways by which hosts are affected (Kammenga et al. 2007). These studies may also provide insight into how hosts adapt to infestation by novel parasites.

Most gene expression studies, to date, have been performed under laboratory conditions with model species, such as *Drosophila melanogaster* and *Arabidopsis thaliana*, both of which are easy to maintain and have fully sequenced genomes (Schenk et al. 2000, Wertheim et al. 2005). Ecologically based studies of gene expression in other systems have increased over the past few years (reviewed in Kammenga et al. 2007);

however, these studies typically use seminatural experiments with animals from the agricultural or fisheries industry with problematic ectoparasites (Gonzalez et al. 2007, Harrington et al. 2010, Porto Neto et al. 2011, Tadiso et al. 2011). Such studies are often prompted because the parasites have evolved resistance against pesticides. Identifying the mechanisms underlying host defense allows resistance in the host population to be increased through artificial selection. Similarly, qualifying host gene expression in response to parasites may also be useful for determining how parasites affect wild hosts, and whether these hosts have evolved defenses against their parasites.

Ectoparasites have been shown to cause significant changes in host gene expression related to the immune response (Harrington et al. 2010, Bonneaud et al. 2011, Li et al. 2011). Indeed, many studies on the effects of ectoparasites on host gene expression focus on skin inflammation, rather than overall changes in gene expression (Gonzalez et al. 2007, Porto Neto et al. 2011, Braden et al. 2012). These studies are relevant because increased host inflammation can prevent parasites from feeding on the host. Other studies are still needed on the molecular basis for other defenses to determine why hosts are differentially affected by their parasites. Gene expression studies in ecology can provide information on the effect of parasites on hosts at a molecular level that are not observed in ecological studies.

The recently introduced parasitic nest fly *Philornis downsi* provides one system in which to study the effect of a parasite on different host species at molecular level. Adult flies, which are not parasitic, lay their eggs in the nests of finches and other land birds in the Galápagos (Fessl et al. 2006). Once the fly eggs hatch, the larvae feed on the blood of nestlings and adult females when they sit on the nest. *P. downsi* has been implicated

in the decline of critically endangered species of Darwin's finches, such as the mangrove finch (*Camarhynchus heliobates*) (Fessl et al. 2010). Several studies have shown that *P. downsi* reduces the reproductive success of Darwin's finches (Koop et al. 2011, 2013a, O'Connor et al. 2013) and that they may drive common finch species to local extinction within a century (Appendix A). It appears that finches have not evolved effective defenses against the parasites (Koop et al. 2013a). In contrast, other hosts living at the same location, such as the Galápagos mockingbird (*Mimus parvulus*), are relatively unaffected by *P. downsi* because they are able to tolerate parasite damage (Chapter 3). Although the effect of *P. downsi* on fitness has been extensively studied, the genetic basis of differences in the effect of the parasite on hosts remains unknown.

Studying host gene expression in response to parasites is difficult in the wild for several reasons (Kammenga et al. 2007). First, there are a number of sources of variation among individuals that may affect gene expression, such as age, season, and body condition. Second, experimentally manipulating parasites in wild hosts can be logistically difficult. In the Galápagos host- *P. downsi* system, we are able to address many of these concerns. We used experimental and molecular approaches to explore the potential underlying mechanisms that promote differences in the effect of *P. downsi* on finches and mockingbirds. Specifically, we experimentally manipulated *P. downsi* abundance in the nests of mockingbirds and finches (*Geospiza fortis*), and compared gene expression from erythrocytes (birds have nucleated erythrocytes.) In particular, we looked for changes in genes related to host defense, such as inflammation and nutritional status.

## Material and Methods

### Study system

Our study was conducted January-April 2013, on the island of Santa Cruz in the Galápagos Archipelago. Our field site, El Garrapatero, is a 4 x 3 km area in the arid, coastal zone. Galápagos mockingbirds and medium ground finches are both abundant at the site. Mockingbirds build open cup-shaped nests, primarily in giant prickly pear cacti (*Opuntia echios gigantea*) and *Acacia* trees. Their clutch size ranges from 1-5 eggs and females incubate the eggs for 12-13 days (Grant and Grant 1979). Nestlings spend an average of 15 days in the nest, where both the adult females and males feed them. Mockingbirds usually lay one clutch of eggs per breeding season; if the nest fails completely, then they may lay a second clutch. Mockingbirds typically do not reuse nests.

Finches build their dome-shaped nests primarily in giant prickly pear cacti and *Acacia* trees (Grant 1999). Their clutch size ranges from 2-5 eggs and females incubate the eggs for 10-14 days. Nestlings spend an average of 12 days in the nest, where both the adult females and males feed them. In years of favorable weather and food resources, medium ground finches can lay more than one clutch of eggs within a given breeding season, but they do not reuse nests (Grant 1999).

### Experimental manipulation of parasites

To quantify the effect of *P. downsi* on host fitness, experimental "nonparasitized" nests were sprayed with a 1% aqueous permethrin solution (Permectrin™ II). Control "parasitized" nests were sprayed with water. Permethrin has been used in previous studies and is harmless to birds, including newly hatched nestlings (Chapter 3). Nests

were sprayed soon after the first nestling hatched, then again 4-6 days later. Nest contents were removed briefly during the process of treatment (Chapter 3). The nest contents were returned to the nest once it was dry (<10 minutes). Parents were quick to return to the nest following treatment, and no cases of nest abandonment due to treatment were observed for either host species.

Nestlings were banded with numbered monel bands a unique color band combinations when they reached approximately two-thirds of the nestling developmental period (finches: 8-9 days old; mockingbirds: 10-11 days old). Successful fledging was confirmed by identifying birds from their color bands once they had left the nest, as in previous studies (Koop et al. 2011, 2013b, 2013a). After the birds in a nest had fledged or died, the nest was collected and placed in a sealed plastic bag. The number of *P. downsi* in the nests was then quantified as described below.

## Blood collection

Blood was collected from nestlings when they reached two-thirds of the nestling developmental period. A small blood sample (< 30) was collected in a microcapillary tube via brachial venipuncture. Within 6 hours of collection, the sample was spun at 8000 rpm for 10 minutes in a centrifuge. Plasma and red blood cells were separated and placed in 0.5 mL vials; 1.0 mL of Trizol™ (Invitrogen) was added to each red blood cell sample to preserve the RNA. Samples were stored at -20°C at the Charles Darwin Research Station. After being transported to the University of Utah, samples were held at -80 °C until used for the microarray analysis, which is described below.

## Parasite load

Each nest was carefully dissected within 8 hours of collection and *P. downsi* larvae, pupae, and eclosed pupal cases were counted (Koop et al. 2011, 2013a, 2013b). Parasite density, defined as the number of individual parasites per unit of host (Bush et al. 1997), was calculated. Specifically, density was calculated by dividing the number of parasites per nest by the total mass of nestlings in the nest.

## Microarray transcriptome analysis

For each host species, blood samples from three individuals in either parasitized or nonparasitized nests (for a total of 12 samples). Each sample was run separately on in the microarray analysis. Messenger RNA was isolated from Trizol™ for each erythrocyte sample as per the manufacturer's protocol. The mRNA processing and hybridization were performed at the Genomics Core Laboratory, Center for Reproductive Biology, Washington State University, Pullman, WA using standard Affymetrix reagents and protocol. Briefly, mRNA was transcribed into cDNA with random primers, then cRNA was transcribed from the cDNA. Single-stranded sense DNA was then synthesized, which was fragmented and labeled with biotin. Biotin-labeled fragmented ssDNA was then hybridized to Zebra Finch 1.0 ST v1 microarrays containing more than 23,700 transcripts (Affymetrix, Santa Clara, CA, USA). Hybridized chips were scanned on an Affymetrix an Scanner 3000. CEL files containing raw data were then pre-processed and analyzed with Affymetrix Expression Console 1.3.1.187 using a Robust Multiarray Average algorithm. The signals (from an average of 11 different probes for each transcript) were averaged to give a single value.

Lists of differentially expressed genes for each treatment were generated using the following criteria: 1) signal ratio of parasitized-nonparasitized was greater than 1.2 fold change, 2) the mean difference for unlogged signals between parasitized and nonparasitized was greater than 10, and 3) t-test p-values for the difference between parasitized and nonparasitized birds were less than 0.05.

CEL files from this study have been deposited with the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo). For gene annotation, Affymetrix annotation file FinGene-1_0-stv1.na34.taegut1.transcript.csv was used unless otherwise specified. Annotated lists were used to categorize genes into functional categories.

KEGG pathways were also identified to determine the function of differentially expressed genes using the website http://www.genome.jp/kegg/ (Kyoto Encyclopedia for Genes and Genome, Kyoto University, Japan). KEGG pathways are manually drawn pathway maps, which represent the known molecular interactions for metabolism and cellular processes.

## Results

### Parasite load and fledging success

Nests fumigated with permethrin did not contain any parasites for either mockingbirds ($n$ = 3 nests) or finches ($n$ = 3). The sham-fumigated (control) mockingbird nests contained 37, 37, and 96 parasites. The control finch nests contained 11, 18, and 28 parasites. Thus, parasite density for mockingbirds was 0.35, 0.35, and 0.70 parasites per gram of nestling for each of the nests in this study. Similarly, parasite density for finches was 0.28, 0.42, and 0.70 parasites per gram of nestling for each of the

nests in this study. See Chapter 3 for mean parasite abundance and density and fledging success for all mockingbird and finch nests in the study.

## Gene expression

The red blood cell transcriptomes from parasitized and nonparasitized nestlings were compared for mockingbirds and finches. The analysis of the microarray data showed that 598 genes were expressed differently between nonparasitized and parasitized mockingbird nestlings. Of these genes, 273 (45.7%) were down regulated and 325 (54.3%) were up regulated in parasitized birds relative to nonparasitized mockingbirds. Within the total list of differentially expressed genes, 325 (54.3%) were annotated (Table 4.S1). These genes were placed in functional categories using a literature search (Fig. 4.1). Many of the differentially expressed genes contributed to metabolism, signaling, and transcription. Six KEGG pathways contained a significant number of genes that were expressed differently (Table 4.1).

The analysis for finches showed that 321 genes were expressed differently between parasitized and nonparasitized birds. Of these genes, 195 (61.7%) were down regulated and 126 (39.3%) were up regulated in parasitized relative to nonparasitized finches. Within the total list of differentially expressed genes, 156 (48.6%) were annotated (Table 4.S2). These genes were placed into functional categories (Fig. 4.1). Similar to mockingbirds, many of the differentially expressed genes contributed to metabolism, signaling, and transcript. Four KEGG pathways contained a significant number of genes that were expressed differently (Table 4.1).

Only 30 specific genes were expressed differently in both parasitized finches and mockingbirds (Table 4.2). Twenty-one of these genes were annotated and all genes were down regulated in both host species.

Parasitized finches had significantly more down-regulated genes than up-regulated genes, compared to parasitized mockingbirds (Fisher's exact test: $P < 0.0001$). Parasitized mockingbirds had more differentially expressed genes related to development than parasitized finches, although this difference was not statistically significant ($P = 0.11$). In contrast, parasitized finches had more differentially expressed genes related to DNA repair ($P = 0.03$), metabolism and transport ($P = 0.03$), and translation and protein modification ($P = 0.003$) compared to parasitized mockingbirds (Fig. 4.1).

## Discussion

*P. downsi* had a significant effect on gene expression from erythrocytes of mockingbirds and finches. Only 30 of the same genes were expressed differently in both parasitized mockingbirds (5% of all genes) and finches (9% of all genes), suggesting that the two species of hosts have different responses to the parasite. Parasitized mockingbirds had more than double the number of differentially expressed genes than parasitized finches. Mockingbirds had a small number of differentially expressed genes related to an immune response, including one gene related to the antibody response; however, these genes did not play a significant role in KEGG pathways related to the immune system. Instead, *P. downsi* had a significant effect on other physiological processes. Notably, parasitized finches had more differentially expressed genes than parasitized mockingbirds related to DNA repair, metabolism and transport, and translation and protein modification. Our results suggest that *P. downsi* has a significant

effect on host gene expression. Most differentially expressed genes in parasitized birds (compared to nonparasitized birds) differed between mockingbirds and finches. These differences may be related to the different effects of *P. downsi* on mockingbirds and finches.

Mockingbirds had several KEGG pathways significantly affected by *P. downsi* parasitism (Table 4.1). Interestingly, the porphyrin metabolism pathway was affected, which is related to the synthesis of heme. Mockingbird and finch nestlings both suffer significant blood loss to *P. downsi* (Chapter 3). Increased porphyrin metabolism near the time of fledging could conceivably help mockingbirds recover red blood cells faster than finches after they have left the nest. The gonadotropin-releasing hormone (GnRH) pathway, which is responsible for neurohormones involved in reproduction, was also affected. A change in this pathway may result in a change in sexual development. Other affected pathways related to the gap junction, alanine, aspartate and glutamate metabolism, and oocyte meiosis are likely affecting normal cellular function, but the specific effect on the bird is unclear.

Similarly, finches had four KEGG pathways significantly affected by *P. downsi* parasitism (Table 4.1). For example, metabolic-related pathways had 21 genes affected, which could explain why finches suffer reduced growth. Changes in ribosomal pathways, which are critical for protein synthesis (translation) in the cell and disruptions in ribosomal pathways, may have been caused by blood loss and lead to cell cycle arrest (Ferreira-Cerca and Hurt 2009). These results indicate that normal cellular function is disrupted by parasitism, which could have lasting effects on adult birds. It would be

interesting to compare gene expression of adults that are parasitized and not parasitized as nestlings to determine whether *P. downsi* has long-term effects on gene expression.

Finches had significantly more genes expressed that were related to DNA repair, compared to mockingbirds. This suggests that parasitized finches have a large amount of cellular DNA damage, which may affect cell survival and could have lasting effects on host survival. DNA damage can be caused by environmental agents, such as UV-radiation and smoking, as found in humans, but also by oxidative damage from byproducts of metabolic processes (Clancy 2008). Nearly 31% of differentially expressed genes in parasitized finch nestlings were related to metabolic processes (compared to only 16% in mockingbirds), which could contribute to DNA damage and, in turn, repair. High DNA damage that cannot be repaired is associated with cell death and could have effects on nestling development and survival.

Our study provided little evidence of gene expression related to the immune response. In Chapter 3, we found that mockingbird and finch nestlings do not have a detectable antibody response to *P. downsi*. We predicted that other aspects of the immune system, which could not be quantified from live animals in our study (e.g. inflammatory response), would be apparent in our gene expression analysis. However, we did not find evidence of a significant change in expression of immune-related genes. Rosenblum et al. (2009) found that pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*) had a significant effect on frog gene expression related to physiological processes, but did not induce expression of genes related to the immune system. Future studies could determine expression of immune-related genes in parasitized adult female

finches, which have a significant immune response to *P. downsi* (Koop et al. 2013a), to determine the specific genes responsible for the antibody response.

Alternatively, genes related to an inflammatory response may be more apparent in skin cells than in erythrocytes. Nucleated erythrocyte gene expression likely reflects a system-wide effect on the organism. However, genes are differentially regulated in different cell types. For example, changes in gene expression related to skin inflammation have been documented in host skin cells (Gonzalez et al. 2007, Porto Neto et al. 2011, Braden et al. 2012). Spleen cells are usually used to identify gene expression related to the humoral immune response, such as the antibody response (Bonneaud et al. 2011). We were unable to collect other types of samples, such as skin or the spleen, from nestling birds. Quantifying gene expression in specific tissues may provide additional results related to specific immune function.

All of the mockingbird and finch nestlings in our study survived to fledging. The amount of blood required for gene expression analysis could only be collected in older nestlings; too much blood taken from younger parasitized nestlings would have resulted in mortality. Thus, genes that were expressed differently in parasitized birds in our study may be different in nestlings that do not survive to fledging. Future studies should look at gene expression in nestlings that do not survive, as well as multiple time points during the nestling period. Such studies may provide reasons for the high mortality in parasitized finches compared to low mortality in parasitized mockingbirds.

Our study is one of few to document the effect of parasites on host gene expression in the field (Kammenga et al. 2007). In contrast to many host-parasite gene expression studies, we did not find that genes related to the host immune response were

expressed significantly when parasitized. However, we did find that *P. downsi* affects physiological processes, which may have contributed to the ability of the hosts to deal with the parasite. Future gene expression studies on this system could focus on other Galápagos species, such as: 1) other Darwin's finch species affected by *P. downsi* to determine what specific genes are related to this effect, and 2) other hosts that are relatively unaffected by *P. downsi* in order to identify the molecular basis for tolerance. A comparison of host gene expression in response to *Philornis* in the parasite's native range could also provide insight into potentially adaptive responses by the host.

## Acknowledgements

This manuscript will be submitted for publication with Michael K. Skinner, Eric E. Nilsson, Jeb P. Owen, and Dale H. Clayton. We thank the Galápagos National Park, Charles Darwin Research Station for various forms of assistance. We also thank Andrew Bartlow, Sabrina McNew, and Daniela Vargas for assistance in the field. The work was supported by NSF grant DEB-0816877 to DHC and University of Utah GCSC grant and a Frank Chapman Student Research Grant to SAK. SAK was supported by a University of Utah Graduate Research Fellowship.

## References

Atkinson, C. T., and D. A. Lapointe. 2009. Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. Journal of Avian Medicine and Surgery 23:53–63.

Bonneaud, C., S. Balenger, A. Russell, J. Zhang, G. Hill, and S. Edwards. 2011. Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. Proceedings of the National Academy of Sciences 108:7866–7871.

Braden, L. M., D. E. Barker, B. F. Koop, and S. R. M. Jones. 2012. Comparative defense-associated responses in salmon skin elicited by the ectoparasite *Lepeophtheirus salmonis*. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 7:100–109.

Bush, A. O., K. D. Lafferty, J. M. Lotz, and A. W. Shostak. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology 83:575–583.

Clancy, S. 2008. DNA damage & repair: mechanisms for maintaining DNA integrity. Nature Education 1:103.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735–748.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443–449.

Ferreira-Cerca, S., and E. Hurt. 2009. Arrest by ribosome. Nature 459:46–47.

Fessl, B., B. J. Sinclair, and S. Kleindorfer. 2006. The life-cycle of *Philornis downsi* (Diptera: Muscidae) parasitizing Darwin's finches and its impacts on nestling survival. Parasitology 133:739–747.

Fessl, B., G. H. Young, R. P. Young, J. Rodríguez-Matamoros, M. Dvorak, S. Tebbich, and J. E. Fa. 2010. How to save the rarest Darwin's finch from extinction: the mangrove finch on Isabela Island. Philosophical Transactions of the Royal Society of London B 365:1019–1030.

Gonzalez, S. F., M. O. Huising, R. Stakauskas, M. Forlenza, B. M. Lidy Verburg-van Kemenade, K. Buchmann, M. E. Nielsen, and G. F. Wiegertjes. 2007. Real-time gene expression analysis in carp (*Cyprinus carpio L.*) skin: inflammatory responses to injury mimicking infection with ectoparasites. Developmental and Comparative Immunology 31:244–254.

Grant, P. 1999. Ecology and Evolution of Darwin's finches. Princeton University Press, Princeton, New Jersey.

Grant, P. R., and N. Grant. 1979. Breeding and feeding of Galápagos Mockingbirds *Nesomimus parvulus*. Auk 96:723–736.

Harrington, D., K. Robinson, J. Guy, and O. Sparagano. 2010. Characterization of the immunological response to *Dermanyssus gallinae* infestation in domestic fowl. Transboundary and Emerging Diseases 57:107–110.

Jetz, W., D. S. Wilcove, and A. P. Dobson. 2007. Projected impacts of climate and land-use change on the global diversity of birds. PLoS Biology 5:e157.

Kammenga, J. E., M. a Herman, N. J. Ouborg, L. Johnson, and R. Breitling. 2007. Microarray challenges in ecology. Trends in Ecology and Evolution 22:273–279.

Keesing, F., L. K. Belden, P. Daszak, A. Dobson, C. D. Harvell, R. D. Holt, P. Hudson, A. Jolles, K. E. Jones, C. E. Mitchell, S. S. Myers, T. Bogich, and R. S. Ostfeld. 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468:647–652.

Koop, J. A. H., J. P. Owen, S. A. Knutie, M. A. Aguilar, and D. H. Clayton. 2013a. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecology and Evolution 3:2514–2523.

Koop, J. A., S. K. Huber, S. M. Laverty, and D. H. Clayton. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PloS One 6:e19706.

Koop, J. A., C. LeBohec, and D. H. Clayton. 2013b. Dry year does not reduce invasive parasitic fly prevalence or abundance in Darwin's finch nests. Reports in Parasitology 3:11–17.

Li, Y. W., X. M. Dan, T. W. Zhang, X. C. Luo, and A. X. Li. 2011. Immune-related genes expression profile in orange-spotted grouper during exposure to *Cryptocaryon irritans*. Parasite Immunology 33:679–687.

O'Connor, J. A., J. Robertson, and S. Kleindorfer. 2013. Darwin's finch begging intensity does not honestly signal need in parasitised nest. Ethology 119:1–10.

Pimm, S. L., C. N. Jenkins, R. Abell, T. M. Brooks, J. L. Gittleman, L. N. Joppa, P. H. Raven, C. M. Roberts, and J. O. Sexton. 2014. The biodiversity of species and their rates of extinction, distribution, and protection. Science 344:987–997.

Porto Neto, L. R., N. N. Jonsson, M. J. D'Occhio, and W. Barendse. 2011. Molecular genetic approaches for identifying the basis of variation in resistance to tick infestation in cattle. Veterinary Parasitology 180:165–172.

Rosenblum, E. B., T. J. Poorten, M. Settles, G. K. Murdoch, J. Robert, N. Maddox, and M. B. Eisen. 2009. Genome-wide transcriptional response of *Silurana (Xenopus) tropicalis* to infection with the deadly chytrid fungus. PloS One 4:e6494.

Schenk, P. M., K. Kazan, I. Wilson, J. P. Anderson, T. Richmond, S. C. Somerville, and J. M. Manners. 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences 97:11655–11660.

Tadiso, T. M., A. Krasnov, S. Skugor, S. Afanasyev, I. Hordvik, and F. Nilsen. 2011. Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. BMC Genomics 12:141.

Vitousek, P. M., C. M. DAntonio, L. L. Loope, M. Rejmanek, and R. Westbrooks. 1997. Introduced species: A significant component of human-caused global change. New Zealand Journal of Ecology 21:1–16.

Wertheim, B., A. R. Kraaijeveld, E. Schuster, E. Blanc, M. Hopkins, S. D. Pletcher, M. R. Strand, L. Partridge, and H. C. J. Godfray. 2005. Genome-wide gene expression in response to parasitoid attack in *Drosophila*. Genome Biology 6:R94.

Table 4.1. KEGG pathways with a significant number of differently expressed (DE) genes in mockingbirds and finches in response to parasitism.

| KEGG pathway | # DE genes in pathway | # genes in pathway | P-value |
|---|---|---|---|
| Mockingbirds | | | |
| Oocyte meiosis | 7 | 90 | 0.008 |
| Adrenergic signaling in cardiomyocytes | 7 | 117 | 0.029 |
| Gap junction | 5 | 75 | 0.041 |
| GnRH signaling pathway | 5 | 72 | 0.035 |
| Alanine, aspartate and glutamate metabolism | 4 | 28 | 0.005 |
| Porphyrin metabolism | 3 | 22 | 0.017 |
| Finches | | | |
| Metabolic pathways | 21 | 935 | 0.031 |
| Ribosome | 9 | 126 | <0.001 |
| Melanogenesis | 4 | 86 | 0.029 |
| ErbB signaling pathway | 4 | 72 | 0.017 |

Table 4.2. Genes that were expressed differently in both medium ground finches and Galápagos mockingbirds in response to parasitism. Twenty-one of 30 genes were annotated, and then classified into functional gene categories. All 21 genes in mockingbirds and finches were down regulated.

| Gene name | Gene symbol | Gene category |
|---|---|---|
| Tubulin beta-6 chain-like | LOC100231628 | Cytoskeleton/Extracellular matrix |
| COP9 constitutive photomorphogenic homolog | COPS2 | Development |
| AMME syndrome gene 1 protein homolog | AMMECR1 | Development |
| Single-stranded DNA binding protein 2 | SSBP2 | Epigenetics |
| O-linked N-acetylglucosamine transferase | OGT | Golgi Apparatus |
| NEFA-interacting nuclear protein-like | LOC100190727 | Growth Factors/Cytokines |
| Cytosolic 5'-nucleotidase III-like | LOC100228312 | Metabolism/Transport |
| Biliverdin reductase A-like | LOC100231163 | Metabolism/Transport |
| ATPase family, AAA domain containing 2B | ATAD2B | Metabolism/Transport |
| Asparagine synthetase (glutamine-hydrolyzing) | ASNS | Metabolism/Transport |
| Potassium channel tetramerisation domain containing 5 | KCTD5 | Metabolism/Transport |
| ATPase, Na+/K+ transporting | ATP1B3 | Metabolism/Transport |
| Ubiquitin specific peptidase 45 | USP45 | Proteolysis |
| Rh-associated glycoprotein | RHAG | Receptors/Binding Proteins |
| KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention | KDELR2 | Receptors/Binding Proteins |
| RAN binding protein 10 | RANBP10 | Receptors/Binding Proteins |
| Inositol-tetrakisphosphate 1-kinase | ITPK1 | Signaling |
| Cytokine inducible SH2-containing protein | CISH | Transcription |
| Nuclear prelamin A recognition factor | NARF | Transcription |
| RNA binding motif protein 38 | RBM38 | Translation/Protein Modification |
| Poly(A) binding protein, cytoplasmic 1 | PABPC1 | Translation/Protein Modification |

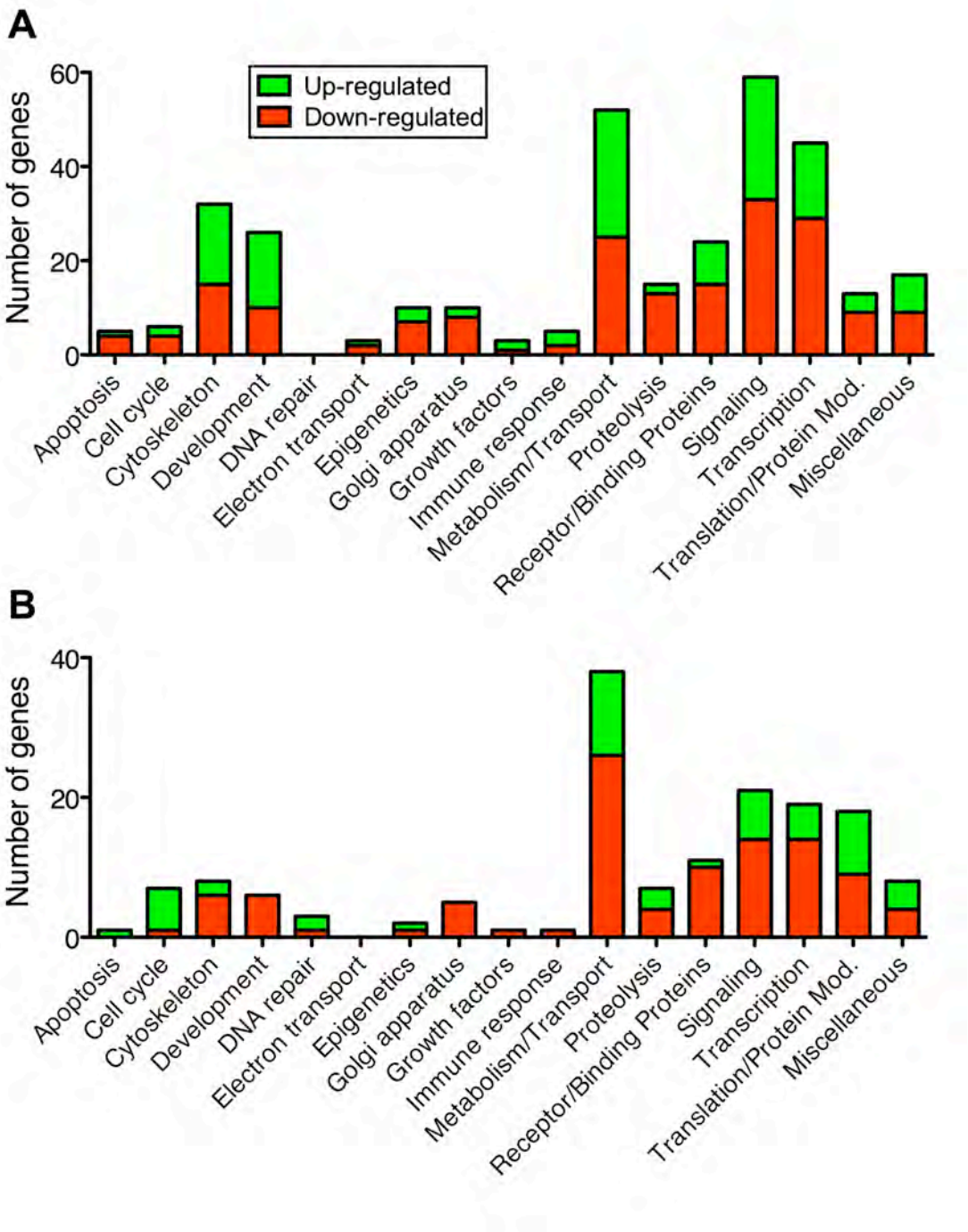


Fig. 4.1. Number of genes with mRNA expression levels in erythrocyte cells that differed significantly between parasitized and nonparasitized A) mockingbird nestlings and B) finch nestlings. Genes were placed in functional categories. Scales differ for each graph.

Table 4.S1. Erythrocyte genes differentially expressed between parasitized and nonparasitized Galapagos mockingbird nestlings as per criteria described in Methods (325 genes annotated). The ratio represents the signal ratio of parasitized to nonparasitized birds. The mean difference is the unlogged signal difference between parasitized and nonparasitized. P-values were calculated from t-tests to compare the difference in gene expression between parasitized and nonparasitized birds.

| Gene Symbol | GeneBank Number | Ratio | Mean Difference | P-value | Affymetrix Probeset ID | Gene Title |
|---|---|---|---|---|---|---|
| **Apoptosis** | | | | | | |
| BFAR | 100223595 | -1.230 | 11.368 | 0.019 | 15627115 | bifunctional apoptosis regulator |
| DDX27 | 100222868 | -1.220 | 10.949 | 0.040 | 15664689 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 27 |
| ING1 | 100232399 | -1.430 | 18.071 | 0.029 | 15600242 | inhibitor of growth family, member 1 |
| LOC100218362 | 100218362 | -2.180 | 84.494 | 0.024 | 15690295 | p53 apoptosis effector related to PMP-22-like |
| TCHP | 100227595 | 1.260 | -13.641 | 0.003 | 15629285 | trichoplein, keratin filament binding |
| **Cell Cycle** | | | | | | |
| CCNY | 100224245 | -1.370 | 40.986 | 0.043 | 15658100 | cyclin Y |
| CEP85L | 100227307 | 1.430 | -21.590 | 0.017 | 15684701 | centrosomal protein 85kDa-like |
| GSPT1 | 100218738 | -1.250 | 41.653 | 0.035 | 15625077 | G1 to S phase transition 1 |
| LOC100226872 | 100226872 | 1.730 | -16.612 | 0.019 | 15740178 | cyclin-A1-like |
| MPHOSPH9 | 100226634 | -1.260 | 14.519 | 0.008 | 15628770 | M-phase phosphoprotein 9 |
| TXLNG | 100224211 | -1.420 | 16.894 | 0.019 | 15605256 | taxilin gamma |
| **Cytoskeleton-Extracellular Matrix** | | | | | | |
| ANKRD27 | 100223380 | -1.420 | 19.058 | 0.024 | 15617332 | ankyrin repeat domain 27 (VPS9 domain) |
| CAPZA1 | 100224610 | -1.560 | 22.645 | 0.022 | 15676365 | capping protein (actin filament) muscle Z-line, alpha 1 |
| CHPF | 100225582 | 1.230 | -10.104 | 0.004 | 15722332 | chondroitin polymerizing factor |
| FGB | 100223598 | 1.490 | -10.372 | 0.046 | 15695368 | fibrinogen beta chain |
| ITM2B | 100221438 | -1.810 | 144.501 | 0.030 | 15607179 | integral membrane protein 2B |
| LGALS8 | 100227037 | -1.260 | 10.629 | 0.041 | 15683510 | lectin, galactoside-binding, soluble, 8 |
| LOC100190164 | 100190164 | 1.220 | -13.112 | 0.049 | 15747117 | clathrin light polypeptide variant 1a-like |
| LOC100190400 | 100190400 | -1.660 | 43.190 | 0.002 | 15666764 | vesicle-associated membrane protein |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100218480 | 100218480 | 1.210 | -31.986 | 0.031 | 15678425 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100218826, LOC100227127, LOC100231293 | 100231293 | 1.210 | -39.043 | 0.034 | 15677594 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100218826, LOC100227127, LOC100231293 | 100231293 | 1.210 | -39.043 | 0.034 | 15677596 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100218826, LOC100227127, LOC100231293 | 100231293 | 1.210 | -39.043 | 0.034 | 15678421 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100219806, LOC100229372 | 100229372 | 1.220 | -22.051 | 0.040 | 15676767 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100221328, LOC100224242 | 100224242 | 1.210 | -32.604 | 0.036 | 15678394 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100221538 | 100221538 | -2.140 | 189.167 | 0.040 | 15658568 | uncharacterized LOC100221538 |
| LOC100222211 | 100222211 | -2.150 | 113.159 | 0.024 | 15746996 | spectrin alpha chain, non-erythrocytic 1-like |
| LOC100222690 | 100222690 | 1.260 | -44.885 | 0.036 | 15676773 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100223342 | 100223342 | -1.390 | 13.727 | 0.042 | 15693337 | microtubule-associated protein RP/EB family member |
| LOC100223683, LOC100232308 | 100232308 | 1.210 | -39.043 | 0.034 | 15676765 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100223683, LOC100232308 | 100232308 | 1.260 | -43.962 | 0.017 | 15676771 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100225573 | 100225573 | 1.230 | -35.822 | 0.035 | 15677602 | feather keratin 2-like |
| LOC100226410, LOC100229942 | 100229942 | 1.250 | -59.315 | 0.038 | 15744834 | feather keratin 1-like, feather keratin 2-like |
| LOC100226559 | --- | 1.210 | -33.576 | 0.031 | 15676769 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| LOC100231628 | 100231628 | -4.000 | 210.104 | 0.031 | 15666718 | tubulin beta-6 chain-like |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100232247 | 100232247 | -1.530 | 46.291 | 0.012 | 15613185 | lamin-L(III)-like |
| LOC100232280 | 100232280 | 1.210 | -58.460 | 0.026 | 15742551 | feather keratin Cos1-1/Cos1-3/Cos2-1-like |
| MTM1 | 100227491 | -1.490 | 14.977 | 0.002 | 15703019 | myotubularin 1 |
| NCDN | 100220891 | 1.310 | -23.983 | 0.027 | 15671977 | neurochondrin |
| RDX | 100228191 | -1.260 | 20.367 | 0.020 | 15606533 | radixin |
| SNAP23 | 100222968 | -1.500 | 153.816 | 0.038 | 15706388 | synaptosomal-associated protein, 23kDa |
| SPTAN1, LOC100227656, LOC100230675 | 100230675 | -1.880 | 151.588 | 0.032 | 15632957 | spectrin, alpha, non-erythrocytic 1, spectrin alpha chain |
| TSPAN5 | 100231192 | -1.520 | 237.840 | 0.047 | 15698341 | tetraspanin 5 |
| **Development** | | | | | | |
| ASAP1 | 100229404 | -1.420 | 34.114 | 0.016 | 15663620 | ArfGAP with SH3 domain, ankyrin repeat |
| CHMP4B | 100219096 | -1.470 | 73.134 | 0.029 | 15665748 | charged multivesicular body protein 4B |
| COPS2 | 100223582 | -1.760 | 29.265 | 0.036 | 15613091 | COP9 constitutive photomorphogenic homolog subunit 2 |
| CPNE3 | 100228795 | -1.390 | 46.959 | 0.021 | 15656473 | copine III |
| DSCR3 | 100222586 | 1.240 | -19.019 | 0.046 | 15604598 | Down syndrome critical region gene 3 |
| DTL | 100227763 | 1.310 | -11.503 | 0.002 | 15687531 | denticleless E3 ubiquitin protein ligase homolog |
| EGLN3 | 100219039 | 1.240 | -16.188 | 0.049 | 15711468 | egl nine homolog 3 (C. elegans) |
| FKTN | 100222184 | 1.270 | -15.869 | 0.029 | 15750483 | fukutin |
| HPS5 | 100222927 | 1.290 | -15.253 | 0.042 | 15709650 | Hermansky-Pudlak syndrome 5 |
| INTS1 | 100223218 | 1.230 | -14.249 | 0.032 | 15625887 | integrator complex subunit 1 |
| LOC100190417 | 100190417 | -1.510 | 271.547 | 0.027 | 15688397 | destrin-like |
| LOC100217631 | 100217631 | -1.880 | 36.353 | 0.025 | 15735826 | luc7-like protein 3-like |
| LOC100223689, | 100228477 | -1.620 | 77.958 | 0.032 | 15702968 | AMME syndrome candidate gene 1 protein homolog |
| LOC100223844 | 100223844 | 1.300 | -17.624 | 0.010 | 15734921 | synaptotagmin-C-like |
| LOC100230241 | 100230241 | -1.600 | 34.731 | 0.046 | 15660304 | retinitis pigmentosa 9 protein-like |
| LOC100230257 | 100230257 | 1.520 | -12.674 | 0.018 | 15746690 | similar to Meckel syndrome, type 1 |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100231601 | 100231601 | -1.430 | 441.301 | 0.006 | 15734987 | similar to MAX interactor 1 |
| LRRC40 | 100232452 | 1.250 | -16.449 | 0.048 | 15726530 | leucine rich repeat containing 40 |
| LRRC8D | 100229262 | -2.010 | 87.422 | 0.045 | 15727572 | leucine rich repeat containing 8 family, member D |
| LRRN1 | 100217548 | 1.350 | -11.345 | 0.006 | 15619547 | leucine rich repeat neuronal 1 |
| LRRTM3 | 100230204 | 1.360 | -19.459 | 0.001 | 15715974 | leucine rich repeat transmembrane neuronal 3 |
| NIPAL4 | 100222378 | 1.260 | -23.751 | 0.047 | 15623415 | NIPA-like domain containing 4 |
| RSAD1 | 100229399 | 1.340 | -23.180 | 0.027 | 15636835 | radical S-adenosyl methionine domain containing 1 |
| SLITRK2 | 100228448 | 1.300 | -10.335 | 0.005 | 15703086 | SLIT and NTRK-like family, member 2 |
| SYT16, LOC100226053 | 100226053 | 1.220 | -13.112 | 0.041 | 15712631 | synaptotagmin XVI, synaptotagmin XVI pseudogene |
| TCTE1 | 100228360 | 1.220 | -20.291 | 0.004 | 15682709 | t-complex-associated-testis-expressed 1 |
| **Electron Transport** | | | | | | |
| LOC100190347 | 100190347 | -1.710 | 37.175 | 0.014 | 15727951 | peroxiredoxin 1-like |
| LOC100224852 | 100224852 | -1.900 | 72.828 | 0.020 | 15618971 | thioredoxin reductase 3-like |
| MTX3 | 100227432 | 1.380 | -10.435 | 0.028 | 15749753 | metaxin 3 |
| **Epigenetics** | | | | | | |
| EPC2 | 100228122 | -1.270 | 13.531 | 0.000 | 15721299 | enhancer of polycomb homolog 2 (Drosophila) |
| JHDM1D | 100223451 | -1.260 | 27.472 | 0.036 | 15645213 | jumonji C domain containing histone demethylase 1 |
| JMJD1C | 100231791 | -1.290 | 35.043 | 0.016 | 15713783 | jumonji domain containing 1C |
| L3MBTL2 | 100218617 | 1.300 | -16.980 | 0.037 | 15648494 | l(3)mbt-like 2 (Drosophila) |
| LOC100224770 | 100224770 | 1.320 | -23.503 | 0.012 | 15600532 | histone H2A.2-like |
| LOC100229097 | 100229097 | -1.420 | 16.432 | 0.013 | 15691024 | histone chaperone ASF1-like |
| MLL3, LOC100230208 | 100230208 | -1.260 | 51.265 | 0.046 | 15657850 | myeloid/lymphoid or mixed-lineage leukemia 3 |
| MSL3 | 100227046 | -1.390 | 14.713 | 0.001 | 15605374 | male-specific lethal 3 homolog (Drosophila) |
| SATB2 | 100229961 | 1.210 | -15.747 | 0.013 | 15720525 | SATB homeobox 2 |
| SSBP2 | 100225156 | -1.880 | 176.559 | 0.020 | 15750626 | single-stranded DNA binding protein 2 |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| **Golgi Apparatus** | | | | | | |
| COG6 | 100224368 | -1.310 | 16.361 | 0.009 | 15601672 | component of oligomeric golgi complex 6 |
| ERO1LB | 100226016 | -1.400 | 21.171 | 0.021 | 15689971 | ERO1-like beta (S. cerevisiae) |
| GALNS | 100222785 | 1.220 | -21.007 | 0.045 | 15616970 | galactosamine (N-acetyl)-6-sulfate sulfatase |
| GORASP2 | 100220731 | -1.540 | 36.280 | 0.038 | 15719878 | golgi reassembly stacking protein 2, 55kDa |
| LOC100227382 | 100227382 | -1.330 | 17.567 | 0.029 | 15642362 | endoplasmic reticulum-Golgi compartment protein 2-like |
| MAN2A1 | 100227845 | 1.400 | -11.950 | 0.043 | 15747882 | mannosidase, alpha, class 2A, member 1 |
| MAN2B2 | 100229436 | -1.330 | 18.186 | 0.005 | 15696822 | mannosidase, alpha, class 2B, member 2 |
| OGT | 100224237 | -1.990 | 242.179 | 0.035 | 15703524 | O-linked N-acetylglucosamine (GlcNAc) transferase |
| SEC24B | 100232172 | -1.420 | 28.488 | 0.014 | 15694999 | SEC24 family, member B (S. cerevisiae) |
| SEC24C | 100224134 | -1.330 | 30.586 | 0.043 | 15716834 | SEC24 family, member C (S. cerevisiae) |
| **Growth Factors & Cytokines** | | | | | | |
| CREG1 | 100221366 | 1.320 | -888.225 | 0.042 | 15603961 | cellular repressor of E1A-stimulated genes 1 |
| LOC100190727 | 100190727 | -1.250 | 19.840 | 0.026 | 15616134 | NEFA-interacting nuclear protein-like |
| LOC100223762 | 100223762 | 1.430 | -19.639 | 0.008 | 15750961 | pro-neuregulin-1, membrane-bound isoform-like |
| **Immune Response** | | | | | | |
| CD36 | 100221639 | -1.420 | 10.213 | 0.038 | 15646424 | CD36 molecule (thrombospondin receptor) |
| ILDR2 | 100225267 | 1.210 | -13.997 | 0.047 | 15603974 | immunoglobulin-like domain containing receptor 2 |
| IRF2 | 100218343 | -1.360 | 60.551 | 0.036 | 15699088 | interferon regulatory factor 2 |
| LOC100224845 | 100224845 | 1.220 | -11.899 | 0.029 | 15704565 | similar to CD6 antigen |
| PLAA | 100231266 | 1.250 | -25.633 | 0.011 | 15752019 | phospholipase A2-activating protein |
| **Metabolism & Transport** | | | | | | |
| ALKBH5 | 100228945 | 1.400 | -19.347 | 0.014 | 15627666 | alkB, alkylation repair homolog 5 (E. coli) |
| AMACR | 100217501 | 1.450 | -14.010 | 0.012 | 15752753 | alpha-methylacyl-CoA racemase |
| ARSB | 100228383 | 1.290 | -11.125 | 0.026 | 15749805 | arylsulfatase B |
| ASNS | 100224891 | -1.600 | 22.443 | 0.033 | 15658803 | asparagine synthetase (glutamine-hydrolyzing) |
| ATAD2B | 100225546 | -1.620 | 46.552 | 0.031 | 15692644 | ATPase family, AAA domain containing 2B |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| ATP1B3 | 100228265 | -1.240 | 16.643 | 0.049 | 15730733 | ATPase, Na+/K+ transporting, beta 3 polypeptide |
| BLVRA | 100224786 | -4.690 | 721.144 | 0.027 | 15658992 | biliverdin reductase A |
| CPOX | 100221663 | -1.580 | 31.155 | 0.048 | 15609080 | coproporphyrinogen oxidase |
| CPS1 | 100221389 | 1.380 | -18.469 | 0.045 | 15721011 | carbamoyl-phosphate synthase 1, mitochondrial |
| DCT | 100230498 | 1.230 | -12.234 | 0.006 | 15601122 | dopachrome tautomerase |
| DCTN4 | 100219462 | -1.550 | 50.953 | 0.022 | 15622157 | dynactin 4 (p62) |
| DSE | 100227259 | 1.260 | -18.356 | 0.024 | 15691120 | dermatan sulfate epimerase |
| EXOSC1 | 100221784 | 1.230 | -28.381 | 0.014 | 15714934 | exosome component 1 |
| FAM3B | 100221253 | 1.260 | -12.279 | 0.017 | 15599321 | family with sequence similarity 3, member B |
| FBP2 | 100219012 | 1.470 | -17.783 | 0.027 | 15747493 | fructose-1,6-bisphosphatase 2 |
| GAD1 | 778442 | 1.240 | -10.773 | 0.045 | 15719865 | glutamate decarboxylase 1 (brain, 67kDa) |
| GCLM | 100231189 | -1.490 | 23.664 | 0.018 | 15725152 | glutamate-cysteine ligase, modifier subunit |
| GID8 | 100232497 | 1.250 | -35.002 | 0.028 | 15666577 | GID complex subunit 8 homolog (S. cerevisiae) |
| GLUD1 | 100222270 | -1.280 | 18.649 | 0.023 | 15716480 | glutamate dehydrogenase 1 |
| HBAD | 100190047 | -1.500 | 965.452 | 0.039 | 15626923 | hemoglobin, alpha 2 |
| HMGCR | 100228972 | 1.380 | -24.818 | 0.040 | 15750388 | 3-hydroxy-3-methylglutaryl-CoA reductase |
| KCNN2 | 100230275 | 1.280 | -13.134 | 0.045 | 15751990 | potassium intermediate/small calcium-activated channel |
| KCTD5 | 100224150 | -1.440 | 39.353 | 0.038 | 15626345 | potassium channel tetramerisation domain containing 5 |
| LOC100190256 | 100190256 | -1.420 | 16.026 | 0.010 | 15728080 | ATP synthase mitochondrial F1 complex assembly factor |
| LOC100218258 | 100218258 | -9.280 | 566.611 | 0.028 | 15686159 | glutathione S-transferase 3-like |
| LOC100218640 | 100218640 | 1.310 | -10.645 | 0.046 | 15737643 | uncharacterized LOC100218640 |
| LOC100218783 | 100218783 | 1.220 | -14.591 | 0.030 | 15737020 | uncharacterized LOC100218783 |
| LOC100218975 | 100218975 | 1.440 | -19.102 | 0.015 | 15699649 | similar to putative tyrosine recombinase |
| LOC100220289 | 100220289 | 1.380 | -32.976 | 0.042 | 15745033 | similar to pristanoyl acyl-Coenzyme A oxidase 3 |
| LOC100221679 | 100221679 | -1.220 | 13.519 | 0.009 | 15603445 | cytochrome c oxidase copper chaperone-like |
| LOC100222183 | 100222183 | 1.410 | -19.139 | 0.009 | 15615912 | arylamine N-acetyltransferase |
| LOC100227411 | 100227411 | 1.580 | -10.086 | 0.012 | 15753898 | monocarboxylate transporter 2-like |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100227765, NAT10 | 100229679 | 1.260 | -17.008 | 0.008 | 15704389 | N-acetyltransferase 10-like, N-acetyltransferase 10 |
| LOC100228312 | 100228312 | -2.500 | 848.276 | 0.000 | 15660310 | cytosolic 5'-nucleotidase III-like |
| LOC100230132 | 100230132 | 1.430 | -10.895 | 0.041 | 15747551 | plasticity related gene 3-like |
| LOC100230342 | 100230342 | -1.250 | 38.063 | 0.044 | 15676915 | ORM1-like protein 3-like |
| LOC100231163 | 100231163 | -2.490 | 118.747 | 0.041 | 15737064 | biliverdin reductase A-like |
| MAT2B | 100226730 | -1.520 | 66.434 | 0.038 | 15622682 | methionine adenosyltransferase II, beta |
| MSMO1 | 100222623 | 1.300 | -16.330 | 0.036 | 15695571 | methylsterol monooxygenase 1 |
| NAA35 | 100221024 | 1.310 | -11.098 | 0.028 | 15753388 | N(alpha)-acetyltransferase 35, NatC auxiliary subunit |
| NAA50 | 100226501 | 1.320 | -34.629 | 0.041 | 15603298 | N(alpha)-acetyltransferase 50, NatE catalytic subunit |
| PCMT1 | 100229428 | -1.230 | 19.520 | 0.008 | 15690661 | protein-L-isoaspartate (D-aspartate) O-methyltransferase |
| PGM2L1 | 100230941 | -1.430 | 18.071 | 0.025 | 15603749 | phosphoglucomutase 2-like 1 |
| PNPT1 | 100223873 | -1.320 | 10.367 | 0.045 | 15682318 | polyribonucleotide nucleotidyltransferase 1 |
| RPE | 100227197 | 1.250 | -13.645 | 0.030 | 15721003 | ribulose-5-phosphate-3-epimerase |
| SLC12A4 | 100230397 | 1.290 | -14.036 | 0.032 | 15615006 | solute carrier family 12 (potassium/chloride transporters) |
| SLC25A12 | 100225577 | 1.260 | -13.720 | 0.024 | 15722847 | solute carrier family 25 (aspartate/glutamate carrier) |
| SLC30A4 | 100219743 | -1.360 | 23.464 | 0.035 | 15613106 | solute carrier family 30 (zinc transporter) |
| TSTD3 | 100232063 | -1.480 | 25.348 | 0.046 | 15691367 | thiosulfate sulfurtransferase (rhodanese)-like domain |
| TTPAL | 100225753 | -1.310 | 30.956 | 0.045 | 15665988 | tocopherol (alpha) transfer protein-like |
| UROS | 100222432 | -2.430 | 90.158 | 0.043 | 15718356 | uroporphyrinogen III synthase |
| **Miscellaneous & Unknown** | | | | | | |
| 100222618 | 100222618 | 1.330 | -20.746 | 0.037 | 15744028 | --- |
| C20H20orf111 | 100232500 | -1.330 | 15.082 | 0.037 | 15664627 | chromosome 20 open reading frame, human C20orf111 |
| C2H7orf41 | 100222580 | 1.220 | -21.007 | 0.033 | 15660092 | chromosome 2 open reading frame, human C7orf41 |
| C3H6orf58 | 100228547 | 1.250 | -19.567 | 0.042 | 15690901 | chromosome 3 open reading frame, human C6orf58 |
| C5H11orf49 | 100228657 | 1.300 | -18.246 | 0.049 | 15710294 | chromosome 5 open reading frame, human C11orf49 |
| EVA1C | 100218472 | 1.290 | -13.939 | 0.013 | 15603708 | eva-1 homolog C (C. elegans) |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| FAM188A | 100222976 | -2.020 | 112.981 | 0.010 | 15652102 | family with sequence similarity 188, member A |
| FAM78A | 100217662 | -1.780 | 82.712 | 0.028 | 15634745 | family with sequence similarity 78, member A |
| KIAA0922 | 100225485 | -1.330 | 50.350 | 0.022 | 15695335 | KIAA0922 ortholog |
| LOC100190044 | 100190044 | -1.210 | 22.424 | 0.046 | 15630012 | uncharacterized LOC100190044 |
| LOC100218251, LOC100225964 | 100225964 | 1.370 | -16.112 | 0.010 | 15674083 | uncharacterized LOC100218251, LOC100225964 |
| LOC100222794 | 100222794 | -1.290 | 13.985 | 0.047 | 15616757 | uncharacterized LOC100222794 |
| LOC100228523 | 100228523 | -1.390 | 15.599 | 0.038 | 15600647 | uncharacterized LOC100228523 |
| LOC100229052 | 100229052 | 1.260 | -30.027 | 0.042 | 15693221 | uncharacterized LOC100229052 |
| LOC100229340 | 100229340 | -1.470 | 14.147 | 0.009 | 15746099 | uncharacterized LOC100229340 |
| PNISR | 100219634 | -1.220 | 40.024 | 0.031 | 15685226 | PNN-interacting serine/arginine-rich protein |
| WDR66 | 100228511 | 1.210 | -14.414 | 0.000 | 15628907 | WD repeat domain 66 |
| **Proteolysis** | | | | | | |
| CLPX | 100225459 | -1.290 | 58.314 | 0.040 | 15611781 | ClpX caseinolytic peptidase X homolog (E. coli) |
| CUL1 | 100223849 | -1.360 | 36.565 | 0.002 | 15658941 | cullin 1 |
| CUL2 | 100221330 | -1.610 | 26.505 | 0.012 | 15651686 | cullin 2 |
| IDE | 100232369 | -1.290 | 24.608 | 0.025 | 15714731 | insulin-degrading enzyme |
| LOC100190161 | 100190161 | -1.230 | 11.526 | 0.002 | 15625854 | proteasome assembling chaperone 3-like |
| LOC100190537 | 100190537 | -1.430 | 38.469 | 0.044 | 15647942 | ubiquitin-conjugating enzyme E2N-like |
| LOC100218931 | 100218931 | 1.220 | -16.596 | 0.015 | 15643310 | cathepsin E-A-like |
| PSMD12 | 100231683 | -1.350 | 13.952 | 0.043 | 15637561 | proteasome (prosome, macropain) 26S subunit |
| RNF138 | 100230774 | -1.280 | 15.359 | 0.026 | 15655839 | ring finger protein 138, E3 ubiquitin protein ligase |
| TIMP3 | 100230145 | -2.310 | 182.693 | 0.022 | 15648915 | TIMP metallopeptidase inhibitor 3 |
| UBA3 | 100222658 | -1.760 | 93.332 | 0.031 | 15621100 | ubiquitin-like modifier activating enzyme 3 |
| UBE2R2 | 100049550 | 1.240 | -56.763 | 0.014 | 15748548 | ubiquitin-conjugating enzyme E2R 2 |
| USP15 | 100224751 | -1.290 | 32.352 | 0.018 | 15643034 | ubiquitin specific peptidase 15 |
| USP3 | 100226009 | -1.280 | 20.837 | 0.039 | 15610024 | ubiquitin specific peptidase 3 |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| USP45 | 100232138 | -1.240 | 10.754 | 0.018 | 15685207 | ubiquitin specific peptidase 45 |
| **Receptors & Binding Proteins** | | | | | | |
| ATXN2 | 100220881 | -1.540 | 100.612 | 0.001 | 15631032 | ataxin 2 |
| CHRNB3 | 100222759 | 1.240 | -11.316 | 0.003 | 15750923 | cholinergic receptor, nicotinic, beta 3 (neuronal) |
| EPB41L4A | 100229687 | 1.370 | -15.747 | 0.003 | 15751604 | erythrocyte membrane protein band 4.1 like 4A |
| EVL | 100228735 | 1.370 | -25.993 | 0.024 | 15707625 | Enah/Vasp-like |
| KDELR2 | 100219316 | -1.870 | 94.616 | 0.018 | 15628207 | KDELendoplasmic reticulum protein retention receptor 2 |
| LOC100218471 | 100218471 | -1.430 | 34.431 | 0.038 | 15703757 | charged multivesicular body protein 1b-like |
| LOC100218897 | 100218897 | 1.210 | -21.362 | 0.041 | 15739913 | similar to discoidin domain receptor family, member 1 |
| LOC100219631 | 100219631 | -1.610 | 43.250 | 0.026 | 15742162 | uncharacterized LOC100219631 |
| LOC100221221 | 100221221 | -1.270 | 12.365 | 0.019 | 15614011 | uncharacterized LOC100221221 |
| LOC100221794 | 100221794 | 1.260 | -11.080 | 0.005 | 15747162 | purpurin-like |
| LOC100227105 | 100227105 | -1.330 | 16.851 | 0.030 | 15639779 | amyloid beta precursor protein-binding protein 2 |
| LOC100227158 | 100227158 | -1.260 | 10.839 | 0.049 | 15638856 | q subcomponent binding protein |
| LOC100228403 | 100228403 | -1.270 | 56.305 | 0.043 | 15746132 | corepressor interacting with RBPJ 1-like |
| LOC100229343 | 100229343 | 1.310 | -16.136 | 0.047 | 15746090 | gamma-aminobutyric acid receptor subunit gamma-2-like |
| PLXND1 | 100223275 | 1.380 | -11.934 | 0.039 | 15621284 | plexin D1 |
| RANBP10 | 100229870 | -1.750 | 206.662 | 0.010 | 15614495 | RAN binding protein 10 |
| REEP1 | 100230095 | -3.400 | 101.490 | 0.014 | 15700563 | receptor accessory protein 1 |
| REEP5 | 100187704 | 1.280 | -14.531 | 0.001 | 15751628 | receptor accessory protein 5 |
| RHAG | 100221695 | -3.170 | 430.880 | 0.010 | 15692863 | Rh-associated glycoprotein |
| TAX1BP1 | 100219738 | -1.850 | 91.716 | 0.038 | 15653346 | Tax1 (human T-cell leukemia virus type I) binding protein |
| TMEM106B | 100231230 | -1.510 | 24.848 | 0.029 | 15653119 | transmembrane protein 106B |
| TMEM185A | 100221681 | -1.440 | 39.006 | 0.049 | 15703038 | transmembrane protein 185A |
| TMEM255A | 100228434 | 1.330 | -11.670 | 0.038 | 15702805 | transmembrane protein 255A |
| WBP4 | 100217656 | -2.210 | 166.095 | 0.016 | 15607338 | WW domain binding protein 4 |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| **Signaling** | | | | | | |
| ADCY9 | 100231171 | 1.330 | -40.079 | 0.042 | 15624742 | adenylate cyclase 9 |
| ADK | 100230888 | -1.660 | 78.660 | 0.021 | 15716748 | adenosine kinase |
| ARL14EP | 100231001 | -1.490 | 20.043 | 0.005 | 15704109 | ADP-ribosylation factor-like 14 effector protein |
| ARRDC2 | 100219453 | 1.300 | -302.224 | 0.050 | 15679730 | arrestin domain containing 2 |
| ARRDC3 | 100228281 | 2.610 | -217.775 | 0.012 | 15751274 | arrestin domain containing 3 |
| CMTM4 | 100230042 | 1.390 | -20.520 | 0.023 | 15615785 | CKLF-like MARVEL transmembrane domain containing 4 |
| DOK4 | 100224124 | -1.240 | 14.191 | 0.003 | 15614391 | docking protein 4 |
| FBXO8 | 100220247 | -1.530 | 167.016 | 0.046 | 15699010 | F-box protein 8 |
| FKBP5 | 100222047 | 1.210 | -10.263 | 0.030 | 15675403 | FK506 binding protein 5 |
| GCHFR | 100220034 | -1.350 | 26.612 | 0.042 | 15704648 | GTP cyclohydrolase I feedback regulator |
| GNAQ | 100230245 | 1.400 | -19.891 | 0.016 | 15749703 | guanine nucleotide binding protein (G protein) |
| GPR116 | 100226589 | -1.420 | 16.939 | 0.007 | 15686711 | G protein-coupled receptor 116 |
| GPRC5B | 100227098 | 1.430 | -17.822 | 0.014 | 15626197 | G protein-coupled receptor, family C, group 5, member B |
| HSPA4L | 100220950 | 1.350 | -21.742 | 0.040 | 15694111 | heat shock 70kDa protein 4-like |
| INPP5E | 100218296 | 1.290 | -15.410 | 0.027 | 15633680 | inositol polyphosphate-5-phosphatase, 72 kDa |
| IP6K1 | 100225979 | 1.360 | -29.700 | 0.021 | 15618128 | inositol hexakisphosphate kinase 1 |
| ITPK1 | 100222999 | -2.320 | 95.727 | 0.039 | 15712157 | inositol-tetrakisphosphate 1-kinase |
| JAK2 | 100225452 | 1.590 | -86.307 | 0.008 | 15753927 | Janus kinase 2 |
| LOC100190399, CALM2 | 100190529 | -1.780 | 40.427 | 0.041 | 15681123 | calmodulin 1, calmodulin 2 (phosphorylase kinase, delta) |
| LOC100190699 | 100190699 | -3.300 | 395.654 | 0.009 | 15613115 | beta 1 integrin binding protein 2-like |
| LOC100218820 | 100218820 | -1.600 | 36.206 | 0.024 | 15702690 | serine/threonine-protein kinase MST4-like |
| LOC100221668 | 100221668 | -1.820 | 90.193 | 0.048 | 15702679 | serine/threonine-protein kinase MST4-like |
| LOC100223639 | 100223639 | 1.450 | -15.982 | 0.046 | 15742585 | similar to odz, odd Oz/ten-m homolog 4 |
| LOC100223676 | 100223676 | -1.230 | 12.099 | 0.044 | 15637984 | GTP-binding protein Rhes-like |
| LOC100224145 | 100224145 | 1.240 | -31.567 | 0.027 | 15716528 | RPE-retinal G protein-coupled receptor-like |
| LOC100224815 | 100224815 | 1.290 | -13.415 | 0.010 | 15710008 | EF-hand calcium-binding domain-containing protein 4A |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100224911, PRKACB, LOC100227397 | 100227397 | -1.400 | 55.485 | 0.025 | 15727669 | cAMP-dependent protein kinase catalytic subunit beta-like |
| LOC100225159 | 100225159 | -2.060 | 164.156 | 0.011 | 15741786 | similar to casein kinase II alpha subunit |
| LOC100225224 | 100225224 | 1.270 | -27.062 | 0.031 | 15723912 | 5-hydroxytryptamine receptor 5A-like |
| LOC100226244 | 100226244 | 1.470 | -29.496 | 0.031 | 15703580 | rho-related GTP-binding protein RhoG-like |
| LOC100227542 | 100227542 | 1.220 | -14.751 | 0.037 | 15701803 | BTB/POZ domain-containing protein KCTD8-like |
| LOC100229199 | 100229199 | -1.340 | 10.445 | 0.041 | 15742006 | protein arginine N-methyltransferase 1-like |
| LOC100230232 | 100230232 | 1.280 | -16.282 | 0.010 | 15668234 | uncharacterized LOC100230232 |
| LOC100230279 | 100230279 | -1.230 | 39.473 | 0.040 | 15736363 | inositol-trisphosphate 3-kinase A-like |
| LOC100230487 | 100230487 | -1.320 | 28.128 | 0.046 | 15729792 | ras-specific guanine nucleotide-releasing factor RalGPS1 |
| LOC100232656 | 100232656 | -3.900 | 235.793 | 0.006 | 15735978 | similar to mitogen-activated protein kinase 6 |
| LPAR1 | 100223722 | 1.330 | -10.295 | 0.025 | 15751961 | lysophosphatidic acid receptor 1 |
| MAB21L1 | 100222410 | 1.220 | -10.607 | 0.038 | 15606891 | mab-21-like 1 (C. elegans) |
| MAPK12 | 100227993 | -1.700 | 56.179 | 0.029 | 15646901 | mitogen-activated protein kinase 12 |
| MAPK6 | 100218726 | -1.980 | 302.319 | 0.015 | 15612923 | mitogen-activated protein kinase 6 |
| MOB2 | 100226865 | -1.300 | 46.189 | 0.018 | 15705622 | MOB kinase activator 2 |
| PDE6D | 100224065 | -1.450 | 13.254 | 0.015 | 15733303 | phosphodiesterase 6D, cGMP-specific, rod, delta |
| PDIK1L | 100224737 | -2.170 | 156.583 | 0.040 | 15671490 | PDLIM1 interacting kinase 1 like |
| PLCH1 | 100219860 | 1.290 | -13.792 | 0.022 | 15731867 | phospholipase C, eta 1 |
| PPAP2B | 100227047 | 1.210 | -13.827 | 0.043 | 15728498 | phosphatidic acid phosphatase type 2B |
| PPP1R21 | 100231538 | 1.490 | -36.128 | 0.012 | 15681964 | protein phosphatase 1, regulatory subunit 21 |
| PPP2R5C | 100229655 | -2.080 | 84.351 | 0.032 | 15707661 | protein phosphatase 2, regulatory subunit B', gamma |
| PRLR | 100219498 | 1.510 | -25.724 | 0.029 | 15752787 | prolactin receptor |
| PTP4A1 | 100219208 | -1.260 | 14.204 | 0.039 | 15691904 | protein tyrosine phosphatase type IVA, member 1 |
| RAB11A | 100232170 | -3.310 | 67.295 | 0.044 | 15611843 | RAB11A, member RAS oncogene family |
| RAB3GAP1 | 100220383 | -1.330 | 14.260 | 0.049 | 15721141 | RAB3 GTPase activating protein subunit 1 (catalytic) |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| RAB7A | 100231734 | -2.010 | 146.011 | 0.036 | 15620634 | RAB7A, member RAS oncogene family |
| RALB | 100218799 | -1.420 | 28.096 | 0.016 | 15721925 | v-ral simian leukemia viral oncogene homolog B |
| RAP1GAP2 | 100226189 | -1.410 | 24.225 | 0.048 | 15639990 | RAP1 GTPase activating protein 2 |
| S1PR3 | 100219008 | 1.370 | -14.491 | 0.028 | 15751095 | sphingosine-1-phosphate receptor 3 |
| SGK1 | 100219883 | -1.390 | 401.425 | 0.034 | 15684383 | serum/glucocorticoid regulated kinase 1 |
| SNX25 | 100225086 | -1.270 | 16.282 | 0.049 | 15695859 | sorting nexin 25 |
| SSTR3 | 100227283 | 1.240 | -15.130 | 0.044 | 15644641 | somatostatin receptor 3 |
| STAM | 100229539 | -1.740 | 92.037 | 0.001 | 15658554 | signal transducing adaptor molecule |
| **Transcription** | | | | | | |
| ALX4 | 100221903 | 1.310 | -18.664 | 0.027 | 15710188 | ALX homeobox 4 |
| BAZ1A | 100228641 | -1.910 | 181.950 | 0.029 | 15711501 | bromodomain adjacent to zinc finger domain, 1A |
| BCL9 | 100231223 | 1.570 | -47.076 | 0.022 | 15740375 | B-cell CLL/lymphoma 9 |
| CCDC135 | 100231881 | 1.230 | -10.287 | 0.009 | 15616084 | coiled-coil domain containing 135 |
| CISH | 100222098 | -2.560 | 149.236 | 0.046 | 15619990 | cytokine inducible SH2-containing protein |
| FBXL3 | 100219599 | 1.420 | -47.581 | 0.041 | 15607492 | F-box and leucine-rich repeat protein 3 |
| FOXN2 | 100219023 | -1.560 | 51.874 | 0.037 | 15681959 | forkhead box N2 |
| FOXO4 | 100217526 | 1.460 | -99.091 | 0.050 | 15703663 | forkhead box O4 |
| FYN | 100228220 | 1.220 | -12.065 | 0.029 | 15684872 | FYN oncogene related to SRC, FGR, YES |
| GPBP1 | 100221033 | 1.460 | -16.139 | 0.041 | 15749335 | GC-rich promoter binding protein 1 |
| GSG1 | 100232053 | 1.280 | -13.787 | 0.006 | 15648278 | germ cell associated 1 |
| HMGXB4 | 100220566 | 1.270 | -11.067 | 0.036 | 15648901 | HMG box domain containing 4 |
| IKZF1 | 100227671 | -1.880 | 77.995 | 0.035 | 15654906 | IKAROS family zinc finger 1 (Ikaros) |
| KLF3 | 100227579 | -1.630 | 111.946 | 0.033 | 15699759 | Kruppel-like factor 3 (basic) |
| KLHL24 | 100217688 | 1.290 | -39.555 | 0.030 | 15732079 | kelch-like 24 (Drosophila) |
| LMO2 | 100232598 | -1.290 | 46.391 | 0.033 | 15708789 | LIM domain only 2 (rhombotin-like 1) |
| LOC100189991 | 100189991 | -1.990 | 132.514 | 0.027 | 15600377 | transcription factor Dp 1-like |
| LOC100190187 | 100190187 | -1.380 | 19.744 | 0.013 | 15748848 | activated RNA polymerase II transcription cofactor 4-like |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100217820 | 100217820 | -1.230 | 13.707 | 0.027 | 15638332 | ankyrin repeat domain-containing protein 40-like |
| LOC100218238 | 100218238 | -1.230 | 29.586 | 0.026 | 15739642 | F-box/LRR-repeat protein 5-like |
| LOC100220098, LOC100222974 | 100222974 | -2.230 | 132.883 | 0.029 | 15712888 | F-box only protein 34-like |
| LOC100221059 | 100221059 | -1.230 | 49.758 | 0.011 | 15663140 | 14-3-3 protein zeta-like |
| LOC100221420 | 100221420 | 1.260 | -28.253 | 0.009 | 15739451 | zinc finger protein 551-like |
| LOC100221458 | 100221458 | 1.220 | -13.764 | 0.040 | 15632180 | uncharacterized LOC100221458 |
| LOC100222622 | 100222622 | -1.550 | 23.771 | 0.039 | 15624241 | dnaJ homolog subfamily C member 18-like |
| LOC100230408 | 100230408 | -1.890 | 246.476 | 0.012 | 15694158 | la-related protein 1B-like |
| LZTFL1 | 100219700 | 1.490 | -16.854 | 0.041 | 15660293 | leucine zipper transcription factor-like 1 |
| MATR3 | 100232255 | -1.610 | 40.075 | 0.021 | 15624352 | matrin 3 |
| MED1 | 100224593 | 1.220 | -23.309 | 0.019 | 15677734 | mediator complex subunit 1 |
| NARF | 100223625 | -2.130 | 131.117 | 0.032 | 15637957 | nuclear prelamin A recognition factor |
| NR1H3, LOC100229235 | 100229235 | 1.550 | -31.182 | 0.003 | 15710279 | nuclear receptor subfamily 1, group H, member 3 |
| NR2C2 | 100222281 | -1.460 | 26.921 | 0.037 | 15621327 | nuclear receptor subfamily 2, group C, member 2 |
| PAXBP1 | 100230574 | -1.430 | 30.322 | 0.000 | 15604267 | PAX3 and PAX7 binding protein 1 |
| PLAG1 | 100222114 | -3.310 | 151.422 | 0.022 | 15662426 | pleiomorphic adenoma gene 1 |
| PROX1 | 100232600 | 1.310 | -11.252 | 0.038 | 15687456 | prospero homeobox 1 |
| RAX | 100225706 | -1.210 | 10.246 | 0.004 | 15754728 | retina and anterior neural fold homeobox |
| RSF1 | 100221084 | -1.540 | 85.785 | 0.002 | 15602693 | remodeling and spacing factor 1 |
| SPTY2D1 | 100226808 | -1.470 | 56.199 | 0.032 | 15709697 | SPT2, Suppressor of Ty, domain containing 1 |
| TBLIX | 100221758 | -2.240 | 164.736 | 0.043 | 15605443 | transducin (beta)-like 1X-linked |
| TCEB1 | 100225017 | -2.190 | 195.259 | 0.016 | 15662756 | transcription elongation factor B (SIII), polypeptide 1 |
| TOP1 | 100222949 | -1.710 | 81.360 | 0.048 | 15664478 | topoisomerase (DNA) I |
| UPF2 | 100231950 | -1.420 | 21.985 | 0.030 | 15646308 | UPF2 regulator of nonsense transcripts homolog |
| WAC | 100230606 | -1.610 | 144.470 | 0.038 | 15658178 | WW domain containing adaptor with coiled-coil |

Table 4.S1. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| ZBTB34 | 100231708 | -1.290 | 29.467 | 0.012 | 15634042 | zinc finger and BTB domain containing 34 |
| ZFX | 100223238 | -1.220 | 38.393 | 0.041 | 15605064 | zinc finger protein, X-linked |
| **Translation & Protein Modification** | | | | | | |
| EEF1A1 | 100223995 | -2.410 | 587.053 | 0.006 | 15685836 | eukaryotic translation elongation factor 1 alpha 1 |
| EIF2A | 100219802 | 1.340 | -11.510 | 0.023 | 15733997 | eukaryotic translation initiation factor 2A, 65kDa |
| LOC100190211 | 100190211 | 1.260 | -13.177 | 0.029 | 15663053 | ribosomal protein L30-like |
| LOC100190645 | 100190645 | -1.650 | 62.780 | 0.018 | 15663384 | eukaryotic translation initiation factor 3 subunit 3 gamma |
| LOC100190647 | 100190647 | -1.620 | 10.709 | 0.042 | 15601290 | 60S ribosomal protein L21-like |
| LOC100223284, EIF3E | 100223993 | -1.340 | 14.978 | 0.042 | 15663274 | eukaryotic translation initiation factor 3 subunit E-like |
| LOC100224980 | 100224980 | -1.230 | 12.789 | 0.043 | 15702635 | integrator complex subunit 6-A-like |
| PABPC1 | 100223990 | -1.350 | 267.608 | 0.031 | 15663125 | poly(A) binding protein, cytoplasmic 1 |
| RBM38 | 100222924 | -1.360 | 207.947 | 0.042 | 15666798 | RNA binding motif protein 38 |
| RPS12 | 100223758 | -1.940 | 105.336 | 0.046 | 15690777 | ribosomal protein S12 |
| TRA2A | 100225439 | -1.540 | 22.827 | 0.009 | 15659623 | transformer 2 alpha homolog (Drosophila) |
| TRMT61A | 100228664 | 1.450 | -20.831 | 0.043 | 15707876 | tRNA methyltransferase 61 homolog A (S. cerevisiae) |
| UTP15 | 100227083 | 1.390 | -30.253 | 0.002 | 15750331 | UTP15, U3 small nucleolar ribonucleoprotein, homolog |

Table 4.S2. Erythrocyte genes differentially expressed between parasitized and nonparasitized medium ground finch nestlings as per criteria described in Methods (156 genes annotated). The ratio represents the signal ratio of parasitized to nonparasitized birds. The mean difference is the unlogged signal difference between parasitized and nonparasitized. P-values were calculated from t-tests to compare the difference in gene expression between parasitized and nonparasitized birds.

| Gene Symbol | GeneBank Number | Ratio | Mean Difference | P-value | Affymetrix Probeset ID | Gene Title |
|---|---|---|---|---|---|---|
| **Apoptosis** | | | | | | |
| LOC100228529 | 100228529 | 1.240 | -19.418 | 0.034 | 15615525 | pleckstrin homology domain-containing family F |
| **Cell Cycle** | | | | | | |
| OSGIN1 | 100229470 | 1.490 | -171.806 | 0.032 | 15616656 | oxidative stress induced growth inhibitor 1 |
| CDK10 | 100224744 | 1.400 | -40.476 | 0.035 | 15615150 | cyclin-dependent kinase 10 |
| RMND1 | 100226682 | 1.400 | -26.984 | 0.016 | 15684167 | required for meiotic nuclear division 1 homolog |
| CDC34 | 100223307 | 1.230 | -20.776 | 0.018 | 15678893 | cell division cycle 34 |
| LOC100217906 | 100225831 | 1.230 | -32.376 | 0.036 | 15707567 | cyclin-K-like, cyclin K |
| ORC2 | 100226144 | 1.220 | -19.794 | 0.043 | 15720493 | origin recognition complex, subunit 2 |
| DOCK8 | 100225500 | -1.380 | 23.377 | 0.050 | 15754073 | dedicator of cytokinesis 8 |
| **Cytoskeleton-Extracellular Matrix** | | | | | | |
| GPC3 | 100219859 | 1.320 | -26.244 | 0.016 | 15701176 | glypican 3 |
| KRT75 | 100224585 | 1.230 | -16.301 | 0.024 | 15734249 | keratin, type II cytoskeletal 75-like |
| MCOLN3 | 100218399 | -1.210 | 14.922 | 0.003 | 15725398 | mucolipin 3 |
| CLTC | 100228504 | -1.240 | 17.141 | 0.040 | 15629739 | clathrin heavy chain 1-like |
| TPGS2 | 100230926 | -1.240 | 11.871 | 0.012 | 15748539 | tubulin polyglutamylase complex subunit 2 |
| LOC100190279 | 100190279 | -1.260 | 28.441 | 0.018 | 15664289 | dynein light chain 2A cytoplasmic-like |
| LOC100231628 | 100231628 | -1.630 | 86.270 | 0.003 | 15666718 | tubulin beta-6 chain-like |
| LOC100227782 | 100227782 | -1.890 | 15.620 | 0.028 | 15740279 | kinesin-like protein KIF21A-like |
| **Development** | | | | | | |
| LUC7L3 | 100230280 | -1.230 | 17.838 | 0.014 | 15636942 | luc7-like protein 3-like, LUC7-like 3 (S. cerevisiae) |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| MIOS | 100219144 | -1.250 | 21.111 | 0.044 | 15652481 | missing oocyte, meiosis regulator, homolog (Drosophila) |
| COPS2 | 100223582 | -1.290 | 19.174 | 0.038 | 15613091 | COP9 constitutive photomorphogenic homolog subunit 2 |
| LOC100222462 | 100222462 | -1.410 | 15.285 | 0.009 | 15607487 | ES1 protein homolog, mitochondrial-like |
| LOC100223129 | 100223129 | -1.420 | 28.216 | 0.012 | 15751660 | amyloid beta A4 precursor protein-binding family A |
| AMMECR1 | 100228477 | -1.460 | 115.557 | 0.021 | 15702968 | AMME syndrome candidate gene 1 protein homolog |
| **DNA repair** | | | | | | |
| LOC100219714 | 100219714 | 1.340 | -10.373 | 0.026 | 15741514 | DNA polymerase lambda-like |
| RAD51B | 100225762 | 1.290 | -38.741 | 0.025 | 15711047 | RAD51 homolog B (S. cerevisiae) |
| MSH3 | 100220355 | -1.220 | 11.899 | 0.047 | 15754401 | mutS homolog 3 (E. coli) |
| **Epigenetics** | | | | | | |
| SETD1B | 100225647 | 1.550 | -35.288 | 0.027 | 15628887 | histone-lysine N-methyltransferase SETD1B-like |
| SETBP1 | 100228402 | -1.320 | 22.195 | 0.001 | 15752309 | SET binding protein 1 |
| **Golgi Apparatus** | | | | | | |
| ERGIC3 | 100223272 | -1.280 | 22.487 | 0.031 | 15664092 | ERGIC and golgi 3 |
| GCNT1 | 100221628 | -1.280 | 14.036 | 0.024 | 15753528 | glucosaminyl (N-acetyl) transferase 1, core 2 |
| RPN2 | 100221023 | -1.290 | 59.757 | 0.022 | 15664543 | ribophorin II |
| OGT | 100224237 | -1.460 | 172.527 | 0.009 | 15703524 | O-linked N-acetylglucosamine (GlcNAc) transferase |
| B4GALT1 | 100223650 | -1.550 | 16.692 | 0.019 | 15752099 | UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase |
| **Growth Factors & Cytokines** | | | | | | |
| LOC100190727 | 100190727 | -1.240 | 26.526 | 0.021 | 15616134 | NEFA-interacting nuclear protein-like |
| **Immune Response** | | | | | | |
| CD82 | 100228720 | -1.840 | 156.401 | 0.021 | 15705911 | CD82 molecule |
| **Metabolism & Transport** | | | | | | |
| TXNDC9 | 100227571 | 1.930 | -177.074 | 0.023 | 15600601 | thioredoxin domain containing 9 |
| LOC100224776, LOC100231558 | 100231558 | 1.670 | -10.358 | 0.033 | 15724689 | heme-binding protein 2-like |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| GCLC | 100218278 | 1.570 | -57.958 | 0.014 | 15686106 | glutamate-cysteine ligase, catalytic subunit |
| GGPS1 | 100232753 | 1.490 | -27.571 | 0.034 | 15683475 | geranylgeranyl diphosphate synthase 1 |
| LOC100223364 | 100223364 | 1.440 | -15.331 | 0.012 | 15609411 | similar to syntaxin 19 |
| SERAC1 | 100219307 | 1.330 | -24.672 | 0.013 | 15683967 | serine active site containing 1 |
| KCNJ4 | 100221522 | 1.330 | -17.812 | 0.001 | 15644519 | potassium inwardly-rectifying channel, subfamily J |
| PRPS1 | 100231012 | 1.270 | -36.636 | 0.036 | 15702027 | phosphoribosyl pyrophosphate synthetase 1 |
| LOC100220801 | 100220801 | 1.250 | -15.241 | 0.027 | 15616344 | similar to aspartate aminotransferase |
| AKR1A1 | 100220342 | 1.230 | -29.708 | 0.017 | 15725905 | aldo-keto reductase family 1, member A1 |
| LOC100190394 | 100227546 | 1.220 | -11.817 | 0.032 | 15701368 | NADH dehydrogenase 1 alpha subcomplex 1 7.5 kDa-like |
| RDH12 | 100226769 | 1.210 | -14.020 | 0.028 | 15706628 | retinol dehydrogenase 12 (all-trans/9-cis/11-cis) |
| KCTD5 | 100224150 | -1.240 | 55.305 | 0.014 | 15626345 | potassium channel tetramerisation domain containing 5 |
| CBS | 100222166 | -1.250 | 17.757 | 0.045 | 15604797 | cystathionine-beta-synthase |
| ETNK1 | 100217685 | -1.250 | 12.213 | 0.029 | 15649574 | ethanolamine kinase 1 |
| ATAD2B | 100225546 | -1.260 | 30.871 | 0.038 | 15692644 | ATPase family, AAA domain containing 2B |
| KAT2B | 100227387 | -1.270 | 35.956 | 0.026 | 15653442 | K(lysine) acetyltransferase 2B |
| LOC100190221 | 100190221 | -1.270 | 31.302 | 0.046 | 15675878 | ATP synthase subunit b-like |
| LOC100231163 | 100231163 | -1.270 | 30.027 | 0.022 | 15737064 | biliverdin reductase A-like |
| SLC12A2 | 100230871 | -1.270 | 13.531 | 0.006 | 15750576 | solute carrier family 12 |
| ALDH6A1 | 100226750 | -1.300 | 27.969 | 0.018 | 15738769 | methylmalonate-semialdehyde dehydrogenase [acylating] |
| PPAP2A | 100220959 | -1.310 | 23.954 | 0.033 | 15753181 | phosphatidic acid phosphatase type 2A |
| SLC25A38 | 100230243 | -1.310 | 99.883 | 0.004 | 15653798 | solute carrier family 25, member 38 |
| UROD | 100223234 | -1.320 | 37.373 | 0.005 | 15725874 | uroporphyrinogen decarboxylase |
| MOCS1 | 100232601 | -1.360 | 70.228 | 0.009 | 15687218 | molybdenum cofactor synthesis 1 |
| IDNK | 100224913 | -1.400 | 14.065 | 0.046 | 15753434 | idnK, gluconokinase homolog (E. coli) |
| ASNS | 100224891 | -1.400 | 42.342 | 0.014 | 15658803 | asparagine synthetase (glutamine-hydrolyzing) |
| ATP1B3 | 100228265 | -1.400 | 66.442 | 0.040 | 15730733 | ATPase, Na+/K+ transporting, beta 3 polypeptide |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| MTHFD2 | 100222836 | -1.400 | 83.518 | 0.038 | 15670244 | methylenetetrahydrofolate dehydrogenase |
| TRAPPC10 | 100221241 | -1.400 | 56.650 | 0.025 | 15599489 | trafficking protein particle complex 10 |
| NNT | 100228078 | -1.460 | 47.858 | 0.036 | 15749138 | nicotinamide nucleotide transhydrogenase |
| LOC100228312 | 100228312 | -1.480 | 465.868 | 0.031 | 15660310 | cytosolic 5'-nucleotidase III-like |
| LOC100232533 | 100232533 | -1.480 | 10.444 | 0.016 | 15687636 | cytochrome c oxidase subunit 7A-related protein |
| PSAT1 | 100217791 | -1.480 | 16.733 | 0.030 | 15753459 | phosphoserine aminotransferase 1 |
| LOC100226085 | 100226085 | -1.610 | 35.130 | 0.023 | 15740772 | chloride intracellular channel protein 4-like |
| LOC100190357 | 100190357 | -2.070 | 20.511 | 0.023 | 15667032 | H+ transporting F1 ATP synthase epsilon subunit-like |
| SEPP1 | 100231953 | -2.260 | 247.282 | 0.044 | 15753073 | selenoprotein P, plasma, 1 |
| LOC100218895 | 100218895 | -3.730 | 891.461 | 0.031 | 15675510 | translocator protein-like |
| **Miscellaneous** | | | | | | |
| LOC100190570 | 100190570 | 1.550 | -33.850 | 0.043 | 15671547 | small membrane protein 1-like |
| LOC100221391 | 100221391 | 1.400 | -39.234 | 0.043 | 15736618 | similar to mCG16868 |
| LOC100228523 | 100228523 | 1.370 | -20.923 | 0.033 | 15600647 | uncharacterized LOC100228523 |
| C17H9orf78 | 100232081 | 1.260 | -24.419 | 0.048 | 15634713 | chromosome 17 open reading frame, human C9orf78 |
| TMEM60 | 100228292 | -1.240 | 17.592 | 0.046 | 15642119 | transmembrane protein 60 |
| YTHDF1 | 100225795 | -1.280 | 72.056 | 0.017 | 15665158 | YTH domain family, member 1 |
| WDR41 | 100225455 | -1.320 | 16.958 | 0.050 | 15749846 | WD repeat domain 41 |
| LOC100230021 | 100230021 | -1.520 | 35.601 | 0.049 | 15639112 | uncharacterized LOC100230021 |
| **Proteolysis** | | | | | | |
| RNF13 | 100225572 | 1.400 | -44.447 | 0.050 | 15734024 | ring finger protein 13 |
| LOC100219036 | 100228364 | 1.310 | -90.020 | 0.011 | 15734839 | ubiquitin-like protein FUBI-like |
| LOC100228799 | 100228799 | 1.240 | -18.627 | 0.006 | 15603242 | cystatin-A-like |
| USP45 | 100232138 | -1.210 | 12.205 | 0.042 | 15685207 | ubiquitin specific peptidase 45 |
| ADAMTS8 | 100217683 | -1.270 | 16.891 | 0.027 | 15672655 | ADAM metallopeptidase with thrombospondin type 1 |
| LOC100190356 | 100190356 | -1.290 | 35.158 | 0.019 | 15678226 | proteasome subunit beta type 3-like |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| LOC100232421 | 100232421 | -1.400 | 32.312 | 0.021 | 15751170 | cathepsin L1-like |
| **Receptors & Binding Proteins** | | | | | | |
| LOC100229756 | 100229756 | 1.230 | -129.139 | 0.034 | 15675810 | transmembrane protein 183-like |
| LOC100232014 | 100232014 | -1.220 | 16.828 | 0.042 | 15678327 | protein AF-17-like |
| ABCB10 | 100225114 | -1.230 | 12.613 | 0.035 | 15683365 | ATP-binding cassette, sub-family B (MDR/TAP) |
| KDELR2 | 100219316 | -1.260 | 34.492 | 0.008 | 15628207 | KDEL endoplasmic reticulum protein receptor |
| RANBP10 | 100229870 | -1.260 | 87.421 | 0.033 | 15614495 | RAN binding protein 10 |
| ADRA1B | 100218481 | -1.300 | 25.803 | 0.035 | 15623105 | adrenoceptor alpha 1B |
| FABP2 | 100231493 | -1.300 | 11.310 | 0.019 | 15697644 | fatty acid binding protein 2, intestinal |
| SERTM1 | 100219545 | -1.320 | 20.734 | 0.048 | 15601579 | serine-rich and transmembrane domain containing 1 |
| RHAG | 100221695 | -1.410 | 279.838 | 0.008 | 15692863 | Rh-associated glycoprotein |
| LOC100225691 | 100225691 | -1.450 | 89.165 | 0.002 | 15670613 | RH-like protein-like |
| PGRMC2 | 100222943 | -1.620 | 98.414 | 0.015 | 15697799 | progesterone receptor membrane component 2 |
| **Signaling** | | | | | | |
| TBC1D15 | 100222838 | 1.270 | -31.455 | 0.015 | 15643409 | TBC1 domain family, member 15 |
| ANP32E | 100224085 | 1.250 | -18.005 | 0.047 | 15674249 | acidic (leucine-rich) nuclear phosphoprotein 32 family |
| LOC100220456 | 100220456 | 1.250 | -32.454 | 0.049 | 15680655 | ralA-binding protein 1-like |
| CALCR | 100223972 | 1.240 | -14.117 | 0.011 | 15658721 | calcitonin receptor |
| CAMK2B | 100228031 | 1.240 | -15.235 | 0.009 | 15669296 | calcium/calmodulin-dependent protein kinase II beta |
| GRIA3 | 751953 | 1.230 | -15.529 | 0.041 | 15701385 | glutamate receptor, ionotropic, AMPA 3 |
| MAPK8IP1 | 100223904 | 1.220 | -14.957 | 0.003 | 15710390 | mitogen-activated protein kinase 8 interacting protein 1 |
| LOC100217560 | 100217560 | -1.220 | 19.874 | 0.045 | 15734676 | RPE-retinal G protein-coupled receptor-like |
| TESK2 | 100220334 | -1.220 | 22.269 | 0.020 | 15727937 | testis-specific kinase 2 |
| C2CD2 | 100219383 | -1.230 | 30.843 | 0.020 | 15604730 | C2 calcium-dependent domain containing 2 |
| UBN1 | 100227353 | -1.240 | 37.255 | 0.017 | 15626637 | ubinuclein 1 |
| LOC100226213 | 100226213 | -1.240 | 15.664 | 0.029 | 15752356 | phospholipase A2 inhibitor subunit gamma B-like |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| MAP3K9 | 100229586 | -1.240 | 30.471 | 0.037 | 15706492 | mitogen-activated protein kinase kinase kinase 9 |
| SHC3 | 100221851 | -1.280 | 20.693 | 0.023 | 15747346 | SHC transforming protein 3 |
| LOC100221543 | 100221543 | -1.300 | 35.493 | 0.004 | 15738611 | serine/threonine-protein kinase pim-1-like |
| IBTK | 100231744 | -1.310 | 26.578 | 0.037 | 15685652 | inhibitor of Bruton agammaglobulinemia tyrosine kinase |
| LOC100225934 | 100225934 | -1.350 | 35.565 | 0.035 | 15603094 | lysophospholipid acyltransferase 5-like |
| LOC100220319 | 100220319 | -1.390 | 35.099 | 0.050 | 15746884 | phosphoinositide-3-kinase, catalytic, beta polypeptide |
| RTN4 | 100219085 | -1.400 | 93.963 | 0.015 | 15682407 | reticulon 4 |
| ITPK1 | 100222999 | -1.470 | 37.111 | 0.024 | 15712157 | inositol-tetrakisphosphate 1-kinase |
| LOC100190361 | 100190361 | -1.650 | 112.380 | 0.005 | 15705707 | v-Ha-ras Harvey sarcoma viral oncogene-like |
| **Transcription** | | | | | | |
| LOC100220780 | 100220780 | 1.390 | -36.988 | 0.043 | 15692675 | pre-mRNA branch site protein p14-like |
| HINT1 | 751976 | 1.280 | -10.861 | 0.024 | 15751052 | histidine triad nucleotide binding protein 1 |
| NRAS | 100222058 | 1.270 | -40.453 | 0.038 | 15674766 | neuroblastoma RAS viral (v-ras) oncogene homolog |
| LOC100217559 | 100217559 | 1.220 | -18.161 | 0.029 | 15640397 | DNA-directed RNA polymerase II subunit RPB11-like |
| LOC100223224 | 100223224 | 1.220 | -37.972 | 0.038 | 15625928 | AN1-type zinc finger protein 2B-like |
| TBL1XR1 | 100224075 | -1.210 | 31.111 | 0.039 | 15731527 | transducin (beta)-like 1 X-linked receptor 1 |
| ZNF462 | 100222248 | -1.210 | 14.795 | 0.004 | 15750504 | zinc finger protein 462 |
| TLE1 | 100221996 | -1.230 | 20.776 | 0.035 | 15749685 | transducin-like enhancer of split 1 (E(sp1) homolog |
| ZFYVE16 | 100221821 | -1.230 | 14.896 | 0.033 | 15754776 | zinc finger, FYVE domain containing 16 |
| ASB6 | 100225339 | -1.260 | 24.419 | 0.014 | 15634694 | ankyrin repeat and SOCS box containing 6 |
| LOC100229420 | 100229420 | -1.260 | 38.537 | 0.008 | 15629668 | protein HIRA-like |
| MITF | 100232286 | -1.260 | 49.177 | 0.027 | 15619453 | microphthalmia-associated transcription factor |
| IKBKAP | 100229335 | -1.300 | 29.640 | 0.003 | 15748303 | inhibitor of kappa light polypeptide gene enhancer |
| PIAS2 | 100221218 | -1.320 | 56.256 | 0.045 | 15750711 | protein inhibitor of activated STAT, 2 |
| HELZ | 100231759 | -1.340 | 61.137 | 0.014 | 15637533 | helicase with zinc finger |
| HIGD1A | 100231360 | -1.400 | 65.074 | 0.022 | 15651257 | HIG1 hypoxia inducible domain family, member 1A |

Table 4.S2. Continued

| | | | | | | |
|---|---|---|---|---|---|---|
| CISH | 100222098 | -1.460 | 176.152 | 0.021 | 15619990 | cytokine inducible SH2-containing protein |
| NARF | 100223625 | -1.510 | 62.885 | 0.004 | 15637957 | nuclear prelamin A recognition factor |
| LOC100189990 | 100189990 | -1.880 | 59.467 | 0.032 | 15750324 | basic transcription factor 3-like |
| **Translation & Protein Modification** | | | | | | |
| LOC100228364 | 100228364 | 1.470 | -468.681 | 0.039 | 15746811 | 40S ribosomal protein S30-like |
| LOC100219106 | 100219106 | 1.420 | -274.813 | 0.017 | 15741308 | 40S ribosomal protein S5-like |
| LOC100229891 | 100229891 | 1.410 | -101.407 | 0.031 | 15716703 | 40S ribosomal protein S24-like |
| LOC100189961 | 100189961 | 1.370 | -37.794 | 0.034 | 15672802 | 40S ribosomal protein S25-like |
| LOC100231477 | 100231477 | 1.340 | -86.509 | 0.039 | 15647428 | 60S ribosomal protein L18a-like |
| LOC100225758 | 100225758 | 1.260 | -224.128 | 0.049 | 15742944 | 40S ribosomal protein S5-like |
| LUC7L2 | 100219615 | 1.260 | -17.147 | 0.041 | 15644256 | LUC7-like 2 (S. cerevisiae) |
| LOC100232067 | 100232691 | 1.250 | -28.450 | 0.028 | 15633625 | 28S ribosomal protein S2, mitochondrial-like |
| RPL12 | 100223396 | 1.240 | -41.365 | 0.001 | 15632508 | ribosomal protein L12 |
| MRPL34 | 100231505 | -1.230 | 16.301 | 0.043 | 15746515 | mitochondrial ribosomal protein L34 |
| LOC100190308 | 100190308 | -1.260 | 26.907 | 0.003 | 15639040 | mitochondrial ribosomal protein S17-like |
| MRPL50 | 100224449 | -1.290 | 47.366 | 0.036 | 15751211 | mitochondrial ribosomal protein L50 |
| RBM38 | 100222924 | -1.380 | 395.366 | 0.028 | 15666798 | RNA binding motif protein 38 |
| ARL5A | 100219471 | -1.410 | 46.657 | 0.041 | 15724303 | ADP-ribosylation factor-like 5A |
| PABPC1 | 100223990 | -1.430 | 550.889 | 0.033 | 15663125 | poly(A) binding protein, cytoplasmic 1 |
| ARL4A | 100225502 | -1.520 | 44.159 | 0.011 | 15653145 | ADP-ribosylation factor-like 4A |
| SSBP2 | 100225156 | -1.660 | 116.773 | 0.037 | 15750626 | single-stranded DNA binding protein 2 |
| LOC100223356 | 100223356 | -2.090 | 21.077 | 0.006 | 15744385 | H/ACA ribonucleoprotein complex subunit 3-like |

# CHAPTER 5

# COMPARING THE ECOLOGY OF PARASITIC NEST FLIES IN THEIR NATIVE AND NOVEL RANGES


## Abstract

Introduced parasites threaten native host populations in many parts of the world. Naïve hosts often lack effective defenses against introduced parasites. Although this idea is generally accepted, it has seldom been rigorously tested in host-parasite systems. The parasitic nest fly *Philornis downsi* was recently introduced to the Galápagos Islands from mainland South America. *P. downsi* has been implicated in the decline of Darwin's finch populations; however, the parasite has relatively no effect on other host species at the same location, such as the Galápagos mockingbird (*Mimus parvulus*). The goal of our study was to compare effect of native *Philornis* nest flies on hosts in Tobago to the effect of introduced *Philornis* flies on hosts in the Galápagos. In Tobago, we experimentally manipulated native *Philornis trinitensis* abundance in nests of black-faced grassquits (*Tiaris bicolor*) and tropical mockingbirds (*Mimus gilvis*) to compare the effects of the parasite on host reproductive fitness. These Tobago host species are closely related to the Galápagos host species. We then used the same methods to test for the effects of *P. downsi* on Galápagos hosts. Finally, we determined the effectiveness of host antibody response against *Philornis* and surveyed potential enemies of *Philornis* in bird nests. The

number of parasites per gram of nestling was similar across Tobago and Galápagos hosts. *Philornis* had a significant negative effect on grassquit and finch nestling survival, but relatively no effect on either species of mockingbirds. However, fewer nests had parasites in Tobago than in the Galápagos; thus, the overall effect of *Philornis* was lower in Tobago than the Galápagos. Neither Tobago nor Galápagos nestlings had an effective antibody response to reduce parasite load. Parasitoid wasps or ants were not found in control nests in the Galápagos. In Tobago, 30% of nests with *P. trinitensis* had parasitoid wasps and 50% of nests had ants; parasite prevalence also decreased throughout the season. Our study indicates that the top down effects of wasps and ants on parasitic flies may regulate host and native fly population dynamics. Furthermore, introduced *P. downsi* have likely become successful in the Galápagos because they have escaped their natural enemies.

## Introduction

Introduced parasites threaten native host populations in many parts of the world (Vitousek et al. 1997, Daszak et al. 2000, Keesing et al. 2010). One explanation for why introduced parasites are problematic is that naïve hosts lack effective defenses against the parasites. A classic example involves the historical introduction of avian malaria parasites and their mosquito vectors to the Hawaiian Islands. This introduction is thought to have been partly responsible for the extinction of 17 endemic honeycreeper species, which had no defenses against avian malaria (Atkinson and Lapointe 2009). In contrast, some hosts do not suffer the same detrimental effects of malaria because their long-standing relationship with this parasite has selected for effective host defenses within the population (Lachish et al. 2011). While host defense mechanisms may be important for

determining the impact of introduced and native parasites on their hosts, very few comparisons between introduced and native parasite-host systems have been made.

Introduced parasites have colonized the Galápagos Islands of Ecuador in recent decades, threatening endemic species of birds (Wikelski et al. 2004). For example, the introduced parasitic nest fly *Philornis downsi* has been implicated in the decline of critically endangered species of Darwin's finches, such as the mangrove finch (*Camarhynchus heliobates*). Several studies have shown that *P. downsi* reduces the reproductive success of Darwin's finches. In some years, 100% of finch nests at a given location fail to produce fledglings as a direct result of *P. downsi* (Koop et al. 2011, 2013a, O'Connor et al. 2013). In contrast, Galápagos mockingbirds, living in the same location, are relatively unaffected by *P. downsi* because they have effective defense mechanisms to deal with the parasite (Chapter 2); these results contradict the idea that all naïve hosts lack defenses against introduced parasites.

The questions of how and why *P. downsi* became a widespread problem in the Galápagos is not completely understood. To answer these questions, comparative studies of the effects of introduced and native parasites on their hosts are needed. These studies may also help determine whether Galápagos host species have the potential to evolve defenses against *P. downsi.* The goal of our study was to compare the effects of introduced and native *Philornis* parasites on hosts and to survey enemies of *Philornis* in both locations. We tested the effect of native *P. trinitensis* nest flies on black-faced grassquits (*Tiaris bicolor*) and tropical mockingbirds (*Mimis gilvis*) in Tobago; these host species were chosen because the tropical mockingbird is a congener of the Galápagos mockingbird and the black-faced grassquit is in the sister clade of Darwin's finches. We

then compared our results to the effect of introduced *P. downsi* on Darwin's finches and Galápagos mockingbirds in the Galápagos Islands. We also determined the relative importance of host immunological defense against *Philornis*.

## Materials and Methods

### Site and species descriptions

Our study on native *Philornis* parasites was conducted in Tobago from May-July 2012. Tobago is located in the southern Caribbean Sea, 30 km north of Venezuela (11°15' N, 60°40' W). The field site was located in the western coastal lowlands of the island. Black-faced grassquits and tropical mockingbirds are abundant at this site. *Philornis trinitensis* is native to Trinidad and Tobago and is also abundant at this site. Similar to *P. downsi*, adult *P. trinitensis* flies, which are not parasitic, lay their eggs in the nests of land birds (Dodge and Aitken 1968, Couri 1989). Once the fly eggs hatch, the larvae burrow subcutaneously under the skin of nestlings where they feed on blood and other fluids (Fig. 5.1).

Black-faced grassquits build dome-shaped nests primarily in ornamental bushes. Clutch size ranges from 1-5 eggs, and females incubate for 12 days (Restall 2003). Nestlings spend 9-12 days in the nest, prior to fledging. Tropical mockingbirds nest primarily in ornamental bushes and palm trees. Clutch size ranges from 1-4 eggs and females incubate for 13-15 days after which nestlings spend 15 days in the nest (Ffrench 1991).

Our study on the introduced *P. downsi* was conducted on Santa Cruz island in the Galápagos from January-April 2012 (Chapter 2). The Galápagos is located in the Pacific Ocean, 650 km west of mainland Ecuador (0°41' S, 90°13' W). The field site, El

Garrapatero, is in the arid coastal zone. Galápagos mockingbirds and medium ground finches are abundant at the site. *P. downsi* is also abundant at this site (Koop et al. 2011, 2013b, 2013a). Adult *P. downsi* flies, which are not parasitic, lay their eggs in the nests of finches and other land birds in the Galápagos. Once the fly eggs hatch, the larvae feed primarily externally on the blood of nestlings and adult females when they sit on the nest. The first and second instar stage can be subcutaneous (Fessl et al. 2006b). *P. downsi* is a sister taxon of the *angustifrons*-group, which contains *P. trinitensis* (Couri et al. 2007).

Medium ground finches build dome-shaped nests in giant prickly pear cacti (*Opuntia echios gigantea*) or Acacia trees (Grant 1999). Their clutch sizes range from 2-5 eggs and females incubate the eggs for 10-14 days. Nestlings spend an average of 12 days in the nest, where the adult females and males feed them. Galápagos mockingbirds build open cup-shaped nests, primarily in giant prickly pear cacti or Acacia trees. Their clutch size ranges from 1-5 eggs and females incubate the eggs for 12-13 days (Grant and Grant 1979). Nestlings spend an average of 15 days in the nest, where the adult females and males feed them.

## Experimental manipulation of parasites

To quantify the effect of *Philornis* on host fitness, experimental nests were fumigated with a 1% aqueous permethrin solution (Permectrin™ II). Control nests were sham-fumigated with water. Permethrin has been used in previous studies (Fessl et al. 2006a, Koop et al. 2013b, 2013a, O'Connor et al. 2013) and is harmless to birds, including newly hatched nestlings. Nests were sprayed soon after the first nestling hatched, then again 4-6 days later. Nestlings and unhatched eggs were removed during the brief process of treatment. The nest contents were returned to the nest after it was dry

(<10 minutes). Parents quickly returned to the nest after treatment, and no cases of nest abandonment due to treatment were observed for either host species.

Active nests were defined as early, mid, or late in the breeding season (days divided equally among times). For the 49-day season in Tobago, nests were classified as early (May 17-June 1), mid (June 2-17), or late (June 18-July 4). For the 48-day season in the Galápagos, nests were classified as early (February 7-22), mid (February 23-March 9), or late (March 10-25). The beginning and the end of the season were defined as the date of the first and last nest to hatch nestlings.

### Fledging success

Nestlings were uniquely marked with a permanent marker at hatching, then banded with unique color band combinations when they were 9-10 days old. Successful fledging was confirmed by identifying birds once they had left the nest, as in previous studies (Koop et al. 2011, 2013b, 2013a). After the birds in a nest had fledged or died, the nest was collected and placed in a sealed plastic bag. The number of *Philornis* in the nests was then quantified as described below.

### Parasite load

Each nest was carefully dissected within 8 hours of collection and *Philornis* larvae, pupae, and eclosed pupal cases were counted (Koop et al. 2011, 2013b, 2013a). Parasite density, defined as the number of individual parasites per unit of host (Bush et al. 1997), was determined. Specifically, density was calculated by dividing the number of parasites per nest by the total mass of nestlings (within 48 hours of hatching) in that particular nest.

All larvae and pupae were reared to the adult stage to identify the *Philornis* species (Dodge and Aitken 1968). In Tobago, all larvae and pupae were identified as *P. trinitensis*; in the Galápagos, all larvae and pupae were identified as *P. dowsni*. Most larvae were third instars when the nests were collected, which then pupated within 24 hours. Younger larvae, which require a blood meal, died soon after they were collected from the nest so they could not be reared to adulthood. The length (mm) and width (mm) of pupae were also measured with digital calipers. These measurements were used to calculate pupal volume as an estimate of individual parasite size, which is related to lifetime fitness in Muscid flies (Schmidt and Blume 1973, Moon 1980). Adult flies were placed in 95% ethanol for later identification.

Nests with parasitoids reared from *Philornis* pupae were counted. Ants were also surveyed in the nests. Ant collection did not occur until June 16; after this date, a subsample of ants was collected from each nest. All nest fauna were placed in 95% ethanol for later identification.

## Blood sampling

We collected a blood sample (< 30 μl) from each nestling during the second visit to the nest (9-10 days of age). The blood sample was collected in a microcapillary tube via brachial venipuncture. Samples were stored on wet ice in the field. Within 6 hours of collection, samples were spun for 10 minutes in a hand crank centrifuge. Samples were then stored at -20°C until returning to the University of Utah where they were stored at -80°C.

## Immunology

We used enzyme-linked immunosorbent assays (ELISA) to detect the presence of *P. trinitensis*-binding antibodies in the plasma of grassquits and mockingbirds using the protocol from Chapter 2. Ninety-six-well plates were coated with 100 μL/well of *P. downsi* protein extract (capture antigen) diluted in carbonate coating buffer (0.05M, pH 9.6). Plates were incubated overnight at 4°C, then washed and coated with 200 μL/well of bovine serum albumin (BSA) blocking buffer and incubated for 30 minutes at room temperature on an orbital table. Between each of the following steps, plates were washed five times with a Tris-buffered saline wash solution, loaded as described, and incubated for 1 hour on an orbital table at room temperature. Triplicate wells were loaded with 100 μL/well of individual host plasma (diluted 1:100 in sample buffer). Plates were then loaded with 100 μl/well of Goat-αBird-IgG (diluted 1:50,000)(Antibodies Online). Finally, plates were loaded with 100 μL/well of peroxidase substrate (tetramethylbenzidine, TMB: Bethyl Laboratories) and incubated for exactly 30 minutes. The reaction was stopped using 100 μL/well of stop solution (Bethyl Laboratories). Optical density (OD) was measured using a spectrophotometer (BioTek, PowerWave HT, 450-nanometer filter).

On each plate, a positive control of pooled plasma from adult female Darwin's finches was used in triplicate to correct for interplate variation (Koop et al. 2013a). In addition, each plate contained a nonspecific binding (NSB) sample in which capture antigen and detection antibody were added, but plasma was excluded. Finally, each plate included a blank sample in which only the detection antibody was added, but

plasma and capture antigen were excluded. NSB absorbance values were subtracted from the mean OD value of each sample.

## Results

### Parasite load

The experimental treatment of nests with permethrin was effective in eliminating *P. trinitensis* for black-faced grassquits and tropical mockingbirds. Fumigated grassquit nests had zero parasites ($n$ = 20 nests), compared to a mean ± SE of 12.42 ± 4.01 parasites ($n$ = 19) in sham-fumigated nests (Mann-Whitney test, $U = 94.50$, $P < 0.001$). Similarly, fumigated tropical mockingbird nests had zero *P. trinitensis* parasites ($n$ = 18), compared to 36.12 ± 8.80 parasites ($n$ = 17) in sham-fumigated nests ($U = 38.00$, $P < 0.0001$). Parasite abundance for Galápagos hosts was reported in Chapter 2.

Parasite density, measured as the number of parasites per gram of nestling, did not differ significantly among Galápagos and Tobago hosts (Table 5.1). Parasite size, measured as pupal volume, also did not differ significantly among hosts. However, Galápagos hosts had more sham-fumigated nests containing parasites than Tobago hosts (Table 5.1). In Tobago, parasite prevalence differed significantly within the season (Chi-square test, $\chi^2 = 6.79$, $df = 2$, $P = 0.03$); prevalence significantly decreased from mid to late season (Fisher's exact test, $P = 0.02$). *Philornis* parasites were found in 6/9 early-season nests, 11/13 mid-season nests, and 7/18 late-season nests. In the Galápagos, parasite prevalence did not differ significantly within the season ($\chi^2 = 1.44$, $df = 2$, $P = 0.49$). *Philornis* parasites were found in 1/1 early-season nest, 16/17 mid-season nests, and 8/10 late-season nests.

## Fledging success

Sham-fumigated nests with at least one parasite were used to compare fledging success because *Philornis* prevalence was low in Tobago nests. In Tobago, grassquit nests from the fumigated treatment fledged more total nestlings than sham-fumigated nests with parasites (Fig. 5.2). In fumigated grassquit nests, 39 out of 51 (76%) young fledged, compared to 25 out of 49 (51%) young fledged from sham-fumigated nests. In contrast, the total number of tropical mockingbird nestlings that fledged did not differ significantly between fumigated nests and sham-fumigated nests with parasites (Fig. 5.2). In fumigated mockingbird nests, 25 out of 40 (63%) young fledged, compared to 16 out of 39 (41%) young fledged from sham-fumigated nests.

In the Galápagos, finch nests from fumigated nests fledged more total nestlings than sham-fumigated nests with parasites (Fig. 5.2). In fumigated finch nests, 37 out of 43 (86%) young fledged, compared to 13 out of 25 (52%) young fledged from sham-fumigated nests with parasites. In contrast, the number of nestlings that fledged from Galápagos mockingbird nests did not differ between fumigated and sham-fumigated nests with parasites (Fig. 5.2). In fumigated mockingbird nests, 39 out of 51 (77%) young fledged, compared to 42 out of 54 (78%) young fledged from sham-fumigated nests with parasites.

Comparing nests rather than nestlings provided similar results. In Tobago, the number of grassquit nests that fledged at least one young was significantly higher in fumigated nests compared to sham-fumigated nests with parasites (Fisher's exact test, $P = 0.003$). In fumigated grassquit nests, 19 out of 20 (95%) nests fledged at least one young, compared to 10 out of 19 (53%) sham-fumigated nests. For tropical

mockingbirds, the number of nests that fledged at least one young did not differ significantly between fumigated nests and sham-fumigated nests with parasites ($P$ = 0.16). In fumigated mockingbird nests, 14 out of 18 (78%) nests fledged at least one young, compared to 9 out of 17 (53%) sham-fumigated nests with parasites.

In the Galápagos, the number of finch nests that fledged at least one young was higher in fumigated nests compared to sham-fumigated nests with parasites ($P$ = 0.06). In fumigated finch nests, 11 out of 12 (92%) nests fledged at least one young, compared to 6 out of 12 (50%) sham-fumigated nests. The number of mockingbird nests that fledged at least one young did not differ significantly between fumigated nests and sham-fumigated nests with parasites ($P$ = 1.00). In fumigated Galápagos mockingbird nests, 14 out of 16 (81%) nests fledged at least one young, compared to 14 out of 16 (81%) sham-fumigated nests with parasites.

In Tobago, the number of total active nests increased from the early to late part of the season (Chi-square test, $\chi^2 = 10.35$, $df = 2$, $P = 0.006$); there were 19 early-season nests, 25 mid-season nests, and 38 late-season nests. In the Galápagos, the total number of active nests increased from early to mid-season but then decreased late in the season ($\chi^2 = 39.27$, $df = 2$, $P < 0.0001$); there were two early-season nests, 33 mid-season nests, and 21 late-season nests.

## Immunology

Antibody levels were low in Tobago hosts. Antibody levels did not differ significantly between treatments for black-faced grassquits (Mann-Whitney test, $U$ = 56.00, $P$ = 0.21). Mean antibody levels in nestlings from fumigated grassquit nests were 0.06 ± 0.02 ($n$ = 18 nests), compared to 0.10 ± 0.04 ($n$ = 9 nests) in nestlings from sham-

fumigated nests. Similarly, antibody levels did not differ significantly between treatments for tropical mockingbirds (Student's t-test: $t = 1.12$, $df = 21$, $P = 0.28$). Mean antibody levels in nestlings from fumigated tropical mockingbird nests were 0.16 ± 0.02 ($n = 13$ nests), compared to 0.11 ± 0.04 ($n = 10$ nests) in nestlings from sham-fumigated nests. Antibody levels for Galápagos hosts were reported in Chapter 2.

## Other nest fauna

One out of 7 (14%) grassquit nests with *Philornis* contained *Brachymeria* parasitoid wasps, and 4 out of 12 (33%) mockingbird nests contained the same species of wasp. Parasitoid wasps were not found in control nests in the Galápagos. One finch nest contained two *P. downsi* pupal cases with holes that were possibly from wasp eclosure, but the wasps were not recovered to confirm infestation. Tobago had significantly more nests with parasitoid wasps (5/19) than the Galápagos (0/25; Fisher's exact test: $P = 0.01$).

Six species of ants were found in grassquit and mockingbird nests in Tobago. Five out of 10 (50%) grassquit nests contained ants; *Crematogaster rochai, Monomorium floricola, C. limata,* and *Solenopsis* sp. #1 were found in these nests. One grassquit nest had two co-existing species of ants (*C. rochai* and *Solenopsis* sp. #1). Four out of 8 (50%) tropical mockingbird nests contained ants; *C. rochai, M. floricola, C. curvispinosa*, and *Solenopsis* sp. #2 were found in these nests. Two mockingbird nests had two co-existing species of ants (nest #1: *M. floricola* and *C. rochai*; nest #2: *C. curvispinosa* and *Solenospsis* sp. #2). Ant eggs were found in three nests with *M. floricola,* two nests with *C. rochai,* and one nest with *Solenopsis* sp. #2. Ants were not

found in Galápagos bird nests. Tobago had significantly more nests with ants (9/18) than the Galápagos (0/28; Fisher's exact test: $P = 0.0007$).

## Discussion

*Philornis* had a significant effect on fledging success of grassquits and finches, but relatively no effect on mockingbirds. However, fewer nests had parasites in Tobago than in the Galápagos; thus, the overall effect of the parasite was lower in Tobago compared to the Galápagos. Neither Tobago nor Galápagos nestlings had an effective antibody response to reduce *Philornis* load. Parasitoid wasps and ants were not found in the Galápagos. However in Tobago, parasitoid wasps of *P. trinitensis* were found in 30% of nests and ants were found in 50% of nests; these nest fauna may be responsible for the decrease in parasite prevalence throughout the season.

Native *Philornis* flies had a similar effect on Tobago hosts compared to introduced *Philornis* flies in the Galápagos. Several other studies have found that nestlings parasitized with native *Philornis* have lower survival than nonparasitized nests (Arendt 2000, Rabuffetti and Reboreda 2007, Segura and Reboreda 2011, Quiroga and Reboreda 2012, Olah et al. 2013); however, flies were found in less than 50% of nests in these studies. In contrast, *P. downsi* is found in at least 80% of Galápagos host nests and in most years, is found in 100% of nests (Koop et al. 2011, 2013a, Kleindorfer et al. 2014). Thus, the overall effect of *Philornis* on host populations is lower in the native range. However, none of these studies have explained why parasites are found in less than half of host nests.

Tobago nestlings did not have an effective antibody response to reduce *Philornis* flies, which was similar to Galápagos nestlings (Koop et al. 2013a, Chapter 2).

Galápagos mockingbirds are able to tolerate the effects of *P. downsi* with behavioral changes (Chapter 2); parents from parasitized nests feed their nestlings more than nonparasitized nests to compensate for energy lost to the parasite. Because mockingbirds from Tobago are also relatively unaffected by *P. trinitensis*, they may have evolved similar behavioral defenses to deal with the parasite. Tobago hosts may also be avoiding *Philornis* infestation by nesting later in the season to coincide with decreased *Philornis* prevalence. Lower *Philornis* prevalence in Tobago may be because hosts have defenses against parasites that we did not test in our study, such as behavioral or inflammatory responses. Future studies should focus on potential defenses of adult birds in Tobago to determine the importance of host defense in regulating native *Philornis* populations.

Alternatively, top-down predatory pressures may decrease *Philornis* prevalence. One explanation for the success of introduced parasites in a new environment is the enemy release hypothesis (ERH; Keane and Crawley 2002, Liu and Stiling 2006). In this case, introduced parasites spread rapidly because they are liberated from their predators, which normally regulate parasite population dynamics in their native range. The ERH is one of the most cited explanations for the success of introduced plant species that escape their enemies, but only recently has this hypothesis been empirically tested (reviewed in Liu and Stiling 2006). The ERH has also been suggested to be important in animal host-parasite systems. Tobago flies were depredated by a single species of parasitoid wasp in one-third of host nests. In contrast, parasitoids wasps are rarely found in nests in the Galápagos. Other studies have reported 2-5% of Darwin's finch nests have two species of parasitoid wasps (*Spalangia endius* and *Brachymeria podagrica*; Lincago and Causton 2008). Galápagos hosts may experience higher parasite prevalence because parasitoid

wasps are not abundant or the wasps are not highly specific to *P. downsi*. This suggests that introducing a large number of species-specific parasitoid wasps to the Galápagos (i.e. biological control) may reduce *P. downsi* prevalence and alleviate the effect of the parasite on Galápagos hosts. Biological control has been used against agricultural pests for over a century, and has recently been used against introduced species of conservation concern (VanDriesche et al. 2010). Indeed the release of an insect predator to control an introduced herbivore in the Galápagos has been successful (Calderen Alvarez et al. 2012). Thus, biological control may be one of the most viable approaches for the long-term control of the fly in the Galápagos.

Six species of ants were documented in grassquit and mockingbird nests in Tobago. Nearly a century ago, Wheeler (1919) collected eight ant species (not in bird nests) at the same location in Tobago; only one out of eight species was in the same genus (*Solenopsis*) as ants collected in our study. Indeed, *Solenopsis* contains several species of stinging fire ants and most previous studies have focused on the negative effect of fire ants on bird survival (reviewed in Allen et al. 1994). In the Galápagos, Knutie et al. (2014) found that all Darwin's finch nests containing *Solenopsis germinata* (3 out of 20) failed. In Tobago, ants were not responsible for nest failure; neither of the two nests with *Solenopsis* ants failed.

The ants collected in our study are likely commensals that foraged on nest material or possibly *Philornis*. Some bird species preferentially nest near ant nests because the ants appear to protect the birds from predators (Hindwood 1959, Young et al. 1990). *P. trinitensis* numbers were not lower in bird nests with ants, which suggests that ants were not feeding on the larvae. However, ants may have been eating the contents of

*Philornis* pupae. We found that several pupal cases from bird nests with ants had large, unique holes that were not likely made by eclosing flies or parasitoid wasps. Thus, ants may be an additional factor regulating *P. trinitensis* populations, especially given that parasite prevalence decreased throughout the breeding season. Future studies are needed to determine the effects of ants on *Philornis* survival to determine whether ants affect fly populations.

Our study provides some of the first evidence to explain why parasites are problematic in their introduced range. Several studies have demonstrated that plants succeed in their introduced range because they have escaped their herbivore predators (Liu and Stiling 2006); yet, few studies have tested this hypothesis for introduced parasites. We found that native *P. trinitensis* flies in Tobago had significant predatory pressure from wasps and possibly ants to reduce parasite prevalence, which has not been previously reported. Birds are also possibly breeding later in the season to avoid the parasite at its peak abundance. In contrast, *P. downsi* was introduced to the Galápagos without any significant predators; therefore, prevalence remains high in Galápagos nests. Future studies could focus on identifying a *P. downsi*-specific parasitoid wasp species from mainland South America as a candidate for biological control, which may be the best approach to combat *P. downsi* in the Galápagos.

Acknowledgements

This manuscript will be submitted for publication with Jordan H. Herman, Jeb P. Owen, and Dale H. Clayton. We thank the Tobago Department of Natural Resources and the Environment, Mike Rutherford, George Heimpel, Roger Moon, and Ray Martinez for various forms of assistance. We thank Marcia Couri for *Philornis* identification, Jack

Longino for ant species identification, and Gerard Delvare for *Brachymeria* identification. We also thank Tobago Plantations, Mount Irvine Bay Golf Course, Crown Point Beach Hotel, Grafton Beach Resort, Tropikist Beach Hotel, Sunspree Resort Ltd., and Rovanel's Resort for permission to work on their property. Bananaquit Apartments provided discounted housing in Tobago. All procedures were approved by the University of Utah IACUC (protocol #10-07003). The work was supported by NSF grant DEB-0816877 to DHC. SAK was supported by a University of Utah Graduate Research Fellowship.

## References

Allen, C. R., S. Demarais, and R. S. Lutz. 1994. Red imported fire ant impact on wildlife: an overview. The Texas Journal of Science 46:51–59.

Arendt, W. 2000. Impact of nest predators, competitors, and ectoparasites on pearly-eyed thrashers, with comments on the potential implications for Puerto Rican parrot recovery. Ornitologia Neotropical 11:13–63.

Atkinson, C. T., and D. A. Lapointe. 2009. Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. Journal of Avian Medicine and Surgery 23:53–63.

Bush, A. O., K. D. Lafferty, J. M. Lotz, and A. W. Shostak. 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. Journal of Parasitology 83:575–583.

Calderen Alvarez, C., C. E. Causton, M. S. Hoddle, C. D. Hoddle, R. van Driesche, and E. J. Stanek. 2012. Monitoring the effects of *Rodolia cardinalis* on *Icerya purchasi* populations on the Galápagos Islands. BioControl 57:167–179.

Colautti, R. I., A. Ricciardi, I. a. Grigorovich, and H. J. MacIsaac. 2004. Is invasion success explained by the enemy release hypothesis? Ecology Letters 7:721–733.

Couri, M. S. 1989. *Philornis* Meinert, 1890 - Key to adults (Diptera, Muscidae, Reinwardtinae). Revista Brasileira de Zoologia 6:569–576.

Couri, M. S., C. J. B. De Carvalho, and P. Löwenberg-Neto. 2007. Phylogeny of *Philornis* Meinert species (Diptera: Muscidae). Zootaxa:19–26.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443–449.

Dodge, H. R., and T. H. G. Aitken. 1968. *Philornis* Flies from Trinidad (Diptera: Muscidae). Journal of the Kansas Entomological Society 41:134–154.

Fessl, B., S. Kleindorfer, and S. Tebbich. 2006a. An experimental study on the effects of an introduced parasite in Darwin's finches. Biological Conservation 127:55–61.

Fessl, B., B. J. Sinclair, and S. Kleindorfer. 2006b. The life-cycle of *Philornis downsi* (Diptera: Muscidae) parasitizing Darwin's finches and its impacts on nestling survival. Parasitology 133:739–747.

Ffrench, R. 1991. A Guide to the Birds of Trinidad and Tobago. Cornell University Press, New York.

Grant, P. 1999. Ecology and Evolution of Darwin's Finches. Princeton University Press, Princeton, New Jersey.

Grant, P. R., and N. Grant. 1979. Breeding and feeding of Galápagos Mockingbirds *Nesomimus parvulus*. Auk 96:723–736.

Hindwood, K. 1959. The nesting of birds in the nests of social insects. Emu 59:1–36.

Keane, R. M., and M. J. Crawley. 2002. Exotic plant invasions and the enemy release hypothesis. Trends in Ecology and Evolution 17:164–170.

Keesing, F., L. K. Belden, P. Daszak, A. Dobson, C. D. Harvell, R. D. Holt, P. Hudson, A. Jolles, K. E. Jones, C. E. Mitchell, S. S. Myers, T. Bogich, and R. S. Ostfeld. 2010. Impacts of biodiversity on the emergence and transmission of infectious diseases. Nature 468:647–652.

Kleindorfer, S., K. J. Peters, G. Custance, R. Y. Dudaniec, and J. A. O'Connor. 2014. Changes in *Philornis* infestation behavior threaten Darwin's finch survival. Current Zoology 60:542–550.

Koop, J. A. H., J. P. Owen, S. A. Knutie, M. A. Aguilar, and D. H. Clayton. 2013a. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecology and Evolution 3:2514–2523.

Koop, J. A., S. K. Huber, S. M. Laverty, and D. H. Clayton. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PloS One 6:e19706.

Koop, J. A., C. LeBohec, and D. H. Clayton. 2013b. Dry year does not reduce invasive parasitic fly prevalence or abundance in Darwin's finch nests. Reports in Parasitology 3:11–17.

Lachish, S., S. C. L. Knowles, R. Alves, M. J. Wood, and B. C. Sheldon. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. The Journal of Animal Ecology 80:1196–1206.

Lincago, P., and C. Causton. 2008. Crianza en cautiverio de *Philornis downsi*, en las Islas Galápagos. Technical Report, Charles Darwin Research Station, Puerto Ayora, Santa Cruz.

Liu, H., and P. Stiling. 2006. Testing the enemy release hypothesis: a review and meta-analysis. Biological Invasions 8:1535–1545.

Moon, R. 1980. Effects of larval competition on face fly. Environmental Entomology 9:325–330.

O'Connor, J. A., J. Robertson, and S. Kleindorfer. 2013. Darwin's finch begging intensity does not honestly signal need in parasitised nest. Ethology 119:1–10.

Olah, G., G. Vigo, L. Ortiz, L. Rozsa, and D. J. Brightsmith. 2013. *Philornis* sp. bot fly larvae in free living scarlet macaw nestlings and a new technique for their extraction. Veterinary parasitology 196:245–9.

Quiroga, M. A., and J. C. Reboreda. 2012. Lethal and sublethal effects of botfly (*Philornis seguyi*) parasitism on House Wren nestlings. Condor 114:197–202.

Rabuffetti, F. L., and J. C. Reboreda. 2007. Early infestation by bot flies (*Philornis seguyi*) decreases chick survival and nesting success in chalk-browed mockingbirds (*Mimus saturninus*). Auk 124:898–906.

Restall, R. 2003. Breeding in the black-faced grassquit, *Tiaris bicolor omissa*, with notes on behavior. Avicultural Magazine 109:174–179.

Schmidt, C. D., and R. R. Blume. 1973. Laboratory-reared horn flies: relationships between width of head and weight of pupa in both sexes and between these measurements and number of ovarioles in females. Annals of the Entomological Society of America 66:1307–1308.

Segura, L. N., and J. C. Reboreda. 2011. Botfly parasitism effects on nestling growth and mortality of red-crested cardinals. The Wilson Journal of Ornithology 123:107–115.

Torchin, M. E., K. D. Lafferty, A. P. Dobson, V. J. Mckenzie, and A. M. Kuris. 2003. Introduced species and their missing parasites. Nature 421:628–630.

Torchin, M. E., and C. E. Mitchell. 2004. Parasites, pathogens, and invasions by plants and animals. Frontiers in Ecology and the Envionment 2:183–190.

VanDriesche, R. G., R. I. Carruthers, T. Center, M. S. Hoddle, J. Hough-Goldstein, L. Morin, L. Smith, D. L. Wagner, B. Blossey, V. Brancatini, R. Casagrande, C. E. Causton, J. A. Coetzee, J. Cuda, J. Ding, S. V. Fowler, J. H. Frank, R. Fuester, J. Goolsby, M. Grodowitz, T. A. Heard, M. P. Hill, J. H. Hoffmann, J. Huber, M. Julien, M. T. K. Kairo, M. Kenis, P. Mason, J. Medal, R. Messing, R. Miller, A. Moore, P. Neuenschwander, R. Newman, H. Norambuena, W. A. Palmer, R. Pemberton, A. Perez Panduro, P. D. Pratt, M. Rayamajhi, S. Salom, D. Sands, S. Schooler, M. Schwarzlinder, A. Sheppard, R. Shaw, P. W. Tipping, and R. D. van Klinken. 2010. Classical biological control for the protection of natural ecosystems. Biological Control 54:S2–S33.

Vitousek, P. M., C. M. DAntonio, L. L. Loope, M. Rejmanek, and R. Westbrooks. 1997. Introduced species: A significant component of human-caused global change. New Zealand Journal of Ecology 21:1–16.

Wheeler, W. M. 1919. The ants of Tobago Island. Psyche 26:113.

Wikelski, M., J. Foufopoulos, H. Vargas, and H. Snell. 2004. Galápagos birds and diseases: invasive pathogens as threats for island species. Ecology and Society 9:online: http://www.ecologyandsociety.org/vol9/iss1.

Young, B. E., M. Kaspari, and T. E. Martin. 1990. Species-specific nest selection by birds in ant-Acacia trees. Biotropica 22:310–315.

Table 5.1. Comparison of *Philornis* parasite number and size in sham-fumigated nests from Tobago hosts (black-faced grassquit-BFGR and tropical mockingbird-TRMO) and Galápagos hosts (medium ground finch-MGFI and Galápagos mockingbird-GAMO). Numbers represent mean ± SE, except for parasite prevalence, which is the number of nests with parasites out of the total number of nests. Kruskal-Wallis tests were used to compare parasite density and pupal volume across host species and a chi-square test was used to compare parasite prevalence across host species.

| | Tobago hosts | | Galápagos hosts | | |
|---|---|---|---|---|---|
| Parasite parameters | BFGR | TRMO | MGFI | GAMO | Statistics |
| Parasite density | 5.89 ±1.43 (10) | 3.36 ±0.92 (13) | 3.30 ±1.06 (10) | 3.50 ±0.86 (15) | $H$=2.10, $P$=0.55 |
| Pupal volume, $mm^3$ (# of nests) | 78.14 ±13.40 (7) | 107.80 ±8.16 (14) | 108.30 ±6.93 (9) | 115.20 ±6.57 (13) | $H$=5.26, $P$=0.15 |
| Parasite prevalence (# of nests) | 10/19 (53%) (19) | 13/17 (77%) (17) | 10/12 (83%) (12) | 15/16 (94%) (16) | $x^2$=8.53, $df$=3, $P$=0.04 |

Fig. 5.1. Tropical mockingbird nestling with approximately 70 *Philornis trinitensis* parasites [Photo by Jordan Herman].

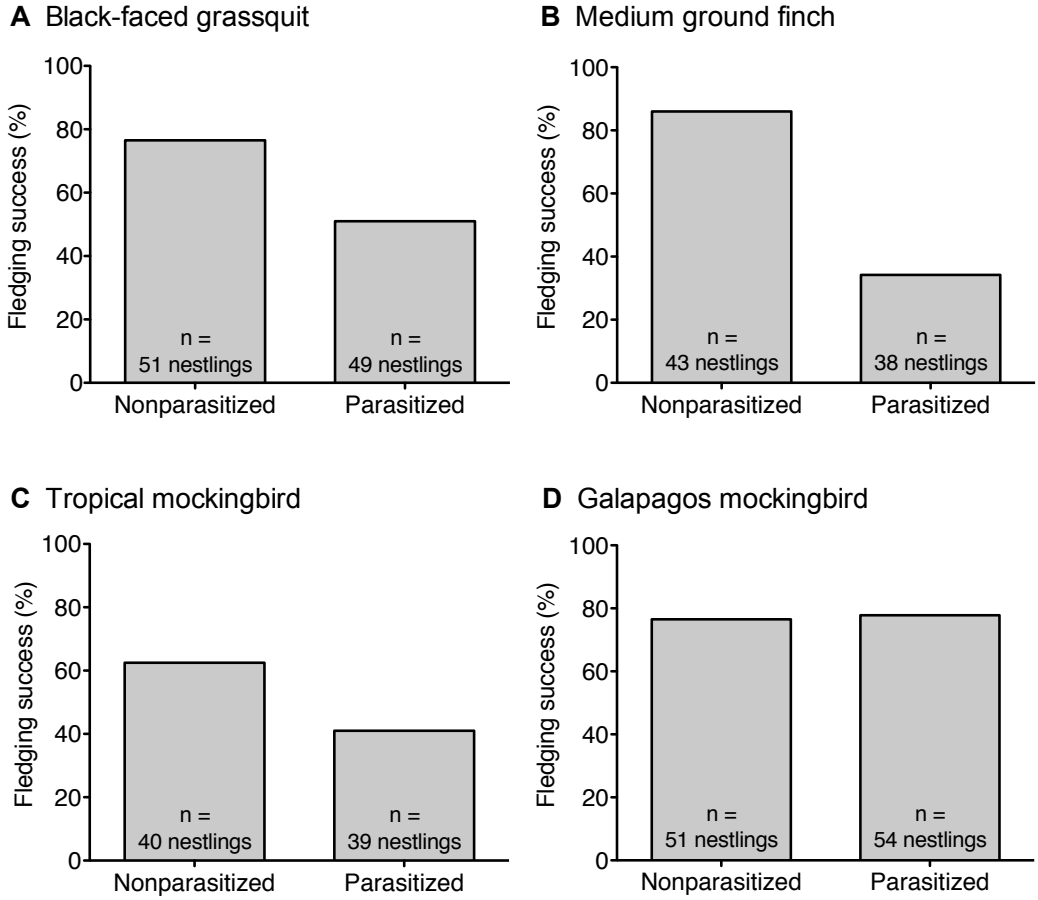


Fig. 5.2. Fledging success of black-faced grassquit (A), medium ground finch (B), tropical mockingbird (C), and Galápagos mockingbird (D) nestlings in fumigated and sham-fumigated nests. Fumigated nests fledged significantly more offspring than sham-fumigated nests for black-faced grassquits (Fisher's exact test, $P = 0.01$) and medium ground finches ($P < 0.0001$). Fledging success did not differ significantly for tropical mockingbirds ($P = 0.07$) or Galápagos mockingbirds ($P = 1.00$).

# CHAPTER 6

# DARWIN'S FINCHES COMBAT INTRODUCED NEST PARASITES WITH FUMIGATED COTTON

Printed with permission from:
Knutie, S.K., S.M. McNew, A.W. Bartlow, D.A. Vargas, and D.H. Clayton. 2014. Darwin's finches combat introduced nest parasites with fumigated cotton. Current Biology 24: R355-R356, doi: 10.1016/j.cub.2014.03.058.

**Magazine**
R355

Correspondence

# Darwin's finches combat introduced nest parasites with fumigated cotton

**Sarah A. Knutie[1,*], Sabrina M. McNew[1], Andrew W. Bartlow[1], Daniela A. Vargas[2] and Dale H. Clayton[1]**

Introduced parasites are a threat to biodiversity when naïve hosts lack effective defenses against such parasites [1]. Several parasites have recently colonized the Galápagos Islands, threatening native bird populations [2]. For example, the introduced parasitic nest fly *Philornis downsi* (Diptera: Muscidae) has been implicated in the decline of endangered species of Darwin's finches, such as the mangrove finch (*Camarhynchus heliobates*) [3]. Here, we show that Darwin's finches can be encouraged to 'self-fumigate' nests with cotton fibers that have been treated with permethrin. Nests with permethrin-treated cotton had significantly fewer *P. downsi* than control nests, and nests containing at least one gram of cotton were virtually parasite-free. Nests directly fumigated with permethrin had fewer parasites and fledged more offspring than nests treated with water.

Adult *P. downsi* flies, which are not parasitic, lay their eggs in the nests of Darwin's finches and other land birds in the Galápagos. Once the eggs hatch, the fly larvae feed on the blood of nestlings and adult females when they sit on the nest. Several previous studies have shown that *P. downsi* reduces the reproductive success of Darwin's finches [4]. In some years, 100% of nests at a given location can fail due to *P. downsi* [4–6]. It is therefore critical that control measures be developed to help reduce the effect of *P. downsi* on endangered Darwin's finches and other birds [3,7].

Our study was conducted January–April, 2013 at the El Garrapatero field site on Santa Cruz island [4,5]. The study was prompted by observations of several species of Darwin's finches incorporating cotton fibers from laundry lines into their nests (Figure 1A). To determine whether finches can be encouraged to self-fumigate their nests, we placed 30 cotton dispensers (Figure 1B) at 40-meter intervals along two transects through our study site (Supplemental information). Preliminary trials showed that finches transport cotton up to 20 meters (Supplemental information).

We used two types of (interspersed) dispensers: experimental dispensers, which contained cotton treated with a 1% permethrin solution, and control dispensers, which contained cotton treated with water. Processed and unprocessed cotton were used to distinguish between the treatments. The two types of cotton were similar in appearance, but could be distinguished upon close inspection. A coin toss determined which treatment was assigned to which cotton type: processed cotton was used for the experimental treatment and unprocessed cotton for the control treatment. A preliminary experiment showed that finches do not discriminate on the basis of cotton type or fumigant (Figure 1C; Supplemental information).

Over the course of the study, we searched once a week for active nests within 20 meters of each dispenser. When a nest was found, it was checked with a camera on a long pole to confirm breeding activity. After the birds finished breeding, the nests were collected and dissected to quantify the number of *P. downsi* in each nest. Cotton and natural nest materials were separated and weighed.

We located 26 active Darwin's finch nests, 22 (85%) of which contained cotton (Figure 1D). None of the nests contained more than one type of cotton. Thirteen nests had experimental (permethrin) cotton and nine nests had control (water) cotton. Nests were constructed by four species of Darwin's finches: *Geospiza fortis, G. fuliginosa*, *Camarhynchus parvulus*, and *Platyspiza crassirostris.* Nests with experimental cotton had a mean (±SE) of 14.69 ± 9.54 parasites; control nests had a mean of 29.89 ± 7.69 parasites (Mann-Whitney test: $U = 31.00$, $P = 0.03$). The effect of the experimental cotton was dose-dependent. Of the eight nests that contained at least one gram of experimental cotton, seven had no parasites and the eighth had only four parasites (Figure 1E). There was no relationship between cotton and parasite load among control nests (Figure 1E).

Monitoring reproductive success requires climbing to nests and banding nestlings, which could interfere with self-fumigation behavior. We therefore quantified the effect of fumigation on host reproductive success using another 37 Darwin's finch nests adjacent to the self-fumigation transects. We sprayed experimental nests with a 1% permethrin solution and control nests with water. Nestlings were banded with color bands, enabling us to confirm fledging success by identifying individual birds after they left the nest [4,5]. Once all of the nestlings in a nest had fledged or died, the nest was collected and dissected to quantify the number of parasites.

The twenty experimental nests sprayed with permethrin had no parasites, while the 17 control nests sprayed with water had a mean of 17.00 ± 3.89 parasites (Mann-Whitney test, $U = 20.00$, $P < 0.0001$). Nineteen of the twenty experimental nests (95%) fledged at least one offspring, while only 11 of the 17 control nests (65%) fledged any offspring (Fisher's exact, $P = 0.03$). Overall, 50 of 60 nestlings (83%) fledged from experimental nests, compared to just 29 of 54 nestlings (54%) from control nests (Figure 1F).

Our study shows that Darwin's finches can control *P. downsi* with permethrin-treated cotton, and that fumigation increases fledging success. There are currently no other effective methods for controlling *P. downsi*. Self-fumigation may thus be a viable approach for combatting *P. downsi* in the nests of Darwin's finches. The mangrove finch is the most critically endangered species of Darwin's finch, with a population of less than 100 individuals restricted to a home range of less than 1 $km^2$ on Isabela Island [3]. Sixty cotton dispensers could treat this entire population. Self-fumigation may be a particularly efficient approach because mangrove finches often build their nests high in mangrove trees, where they are relatively inaccessible [3].

Our study is the first to demonstrate the effectiveness of self-fumigation against parasites. This approach has been tried previously where mice were

**Current Biology** Vol 24 No 9
R356

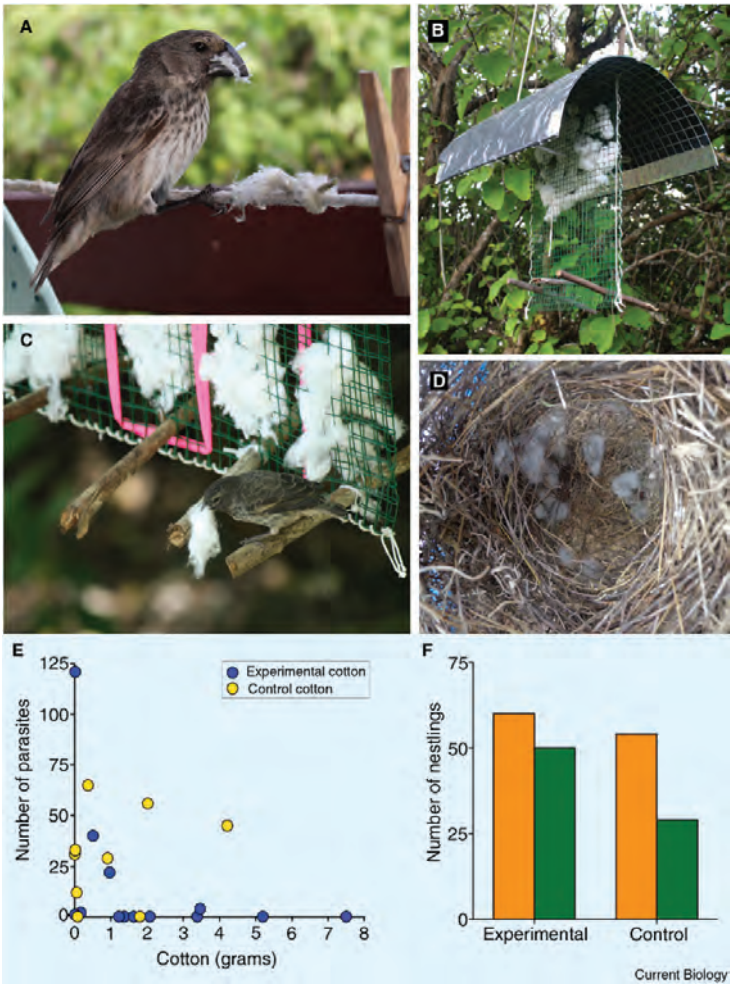


Figure 1. Incorporation of permethrin-treated cotton into nests by Darwin's finches.
(A) Female medium ground finch (*Geospiza fortis*) removing fibers from a cotton laundry line at the Charles Darwin Research Station, Galápagos. (B) Cotton dispenser at the field site; cotton has been removed from the lower half by finches. (C) Small ground finch (*G. fuliginosa*) removing cotton from a dispenser in a preliminary experiment. (D) Finch nest containing about one gram of cotton. (E) Parasite abundance was negatively correlated with the mass of experimental cotton (Spearman rank correlation: $r_s = -0.62$, $P = 0.03$), but not with the mass of control cotton ($r_s = 0.22$, $P = 0.58$). (F) Experimental nests treated with permethrin fledged more offspring than control nests treated with water (Fisher's exact test: $P = 0.001$). Orange bars are the total number of nestlings monitored; green bars are the total number of nestlings that fledged.

encouraged to incorporate fumigated cotton into their nests to kill ticks that vector Lyme disease. However, the effectiveness of the method is not clear [8]. Self-fumigation might also be useful for controlling the fleas that vector plague, which can contribute to the local extinction of black-tailed prairie dogs (*Cynomys ludovicianus*) [9]. Because prairie dogs incorporate plant fibers into their burrows, it might be possible to encourage them to use fumigated materials. Self-fumigation also has potential for the control of parasites in other threatened and endangered bird species. For example, it might be useful for combating explosive increases in lice that appear to have contributed to the decline of the Hawaiian endemic akepa honeycreeper (*Loxops cocineus coccineus*) [10].

**Supplemental Information**

Supplemental Information including experimental procedures and one figure can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.03.058.

**Acknowledgments**

We thank the Galápagos National Park, Charles Darwin Research Station, Fred Adler, Elena Arriero, Emily DiBlasi, Jordan Herman, Michael Thompson and Sarah Windes for assistance. We thank Charles Brown, Sarah Bush, Franz Goller, Jennifer Koop, Cagan Sekercioglu, and two anonymous reviewers for comments on the manuscript. The work was supported by NSF grant DEB-0816877 to D.H.C. and a University of Utah GCSC grant and RocketHub Crowdfunding to S.A.K. We are grateful to U.S. Cotton™ for donating supplies.

**References**

1. Daszak, P., Cunningham, A.A., and Hyatt, A.D. (2000). Emerging infectious diseases of wildlife – threats to biodiversity and human health. Science *287*, 443–449.
2. Wikelski, M., Foufopoulos, J., Vargas, H., and Snell, H. (2004). Galápagos birds and diseases: invasive pathogens as threats for island species. Ecol. Soc. *9*, online: http://www.ecologyandsociety.org/vol9/iss1.
3. Fessl, B., Young, G.H., Young, R.P., Rodríguez-Matamoros, J., Dvorak, M., Tebbich, S., and Fa, J.E. (2010). How to save the rarest Darwin's finch from extinction: the mangrove finch on Isabela Island. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *365*, 1019–1030.
4. Koop, J.A.H., Huber, S.K., Laverty, S.M., and Clayton, D.H. (2011). Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PLoS One *6*, e19706.
5. Koop, J.A.H., Owen, J.P., Knutie, S.A., Aguilar, M.A., and Clayton, D.H. (2013). Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecol. Evol. *3*, 2514–2523.
6. O'Connor, J.A., Robertson, J., and Kleindorfer, S. (2013). Darwin's finch begging intensity does not honestly signal need in parasitised nests. Ethology *119*, 1–10.
7. O'Connor, J.A., Sulloway, F.J., Robertson, J., and Kleindorfer, S. (2010). *Philornis downsi* parasitism is the primary cause of nestling mortality in the critically endangered Darwin's medium tree finch (*Camarhynchus pauper*). Biodivers. Conserv. *19*, 853–866.
8. Ostfeld, R.S., Price, A., Hornbostel, V.L., Benjamin, M.A., and Keesing, F. (2006). Controlling ticks and tick-borne zoonoses with biological and chemical agents. Bioscience *56*, 383–394.
9. Stapp, P., Antolin, M.F., and Ball, M. (2004). Patterns of extinction in prairie dog metapopulations: plague outbreak follow El Niño events. Front. Ecol. Envionment *2*, 235–240.
10. Freed, L.A., Cann, R.L., and Bodner, G.R. (2008). Incipient extinction of a major population of the Hawaii akepa owing to introduced species. Evol. Ecol. Res. *10*, 931–965.

[1]Department of Biology, Univ. of Utah, 257 South 1400 East, Salt Lake City, UT 84112, USA. [2]Facultad de Biosciencias, Univ. Autonoma de Barcelona, Cerdanyola del Valles, 08193 Barcelona, Spain.
*E-mail: saknutie@gmail.com

**Supplemental information**

**Darwin's finches combat introduced nest parasites with fumigated cotton**

**Sarah A. Knutie, Sabrina M. McNew, Andrew W. Bartlow, Daniela A. Vargas, and Dale H.**
**Clayton**

**Supplemental Figure**

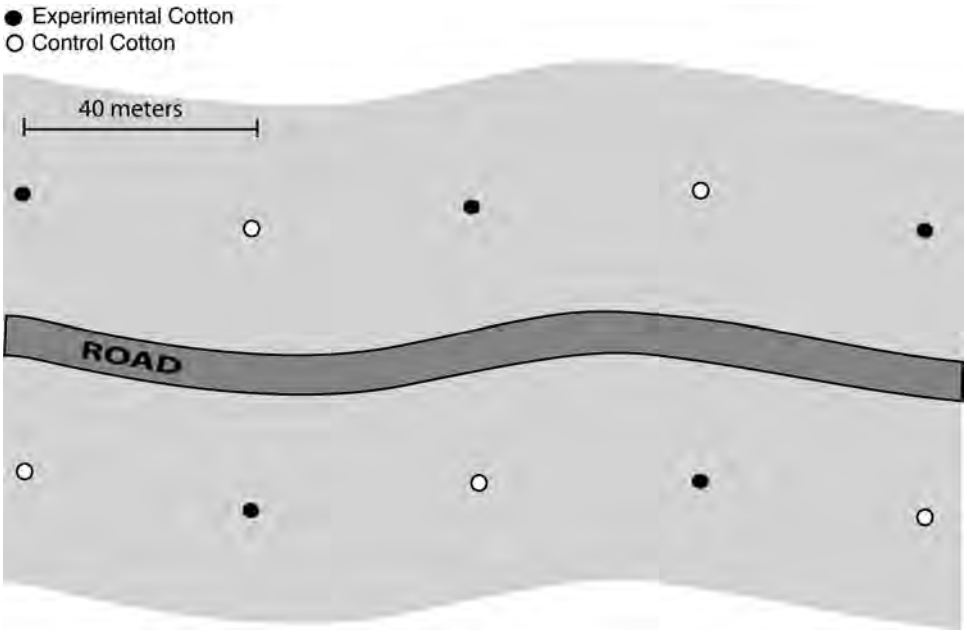


**Figure S1** A partial representation of the field site with cotton dispensers. Light gray area corresponds to the area searched for nests. The experiment had a total of 30 dispensers, with 15 along each side of the road in the pattern shown here.

**Supplemental Experimental Procedures**

**Study system and field site**

Our field site, El Garrapatero, is a 5km x 1.5km area in the southeastern arid coastal zone of Santa Cruz Island, Galápagos. Several species of Darwin's finches are abundant at this site [S1], including the medium ground finch (*Geospiza fortis*), small

ground finch (*G. fuliginosa*), small tree finch (*Camarhynchus parvulus*), and vegetarian finch (*Platyspiza crassirostris*). Finch nests are dome-shaped and constructed mainly of plant fibers. Finches build their nests one to five meters above the ground at this site in endemic tree cacti (*Opuntia echios gigantea*) or *Acacia* trees. All procedures in our study were approved by the University of Utah IACUC (protocol #10-07003).

**Cotton dispensers**

Dispensers were made from 19-gauge hardware cloth, which held cotton in place (Figure 1B). A piece of hardware cloth was folded in half with each side bound together by cotton string along the edges. Two wooden perches were placed approximately 4cm from the bottom of the dispenser. A black plastic roof was attached to the top of each dispenser to slow the degradation of permethrin from exposure to sunlight and rain. Processed and unprocessed cotton were used to distinguish between the experimental and control treatments. Both types of cotton were obtained from U.S. Cotton™. The only difference between the cotton types is that processed cotton is combed to align the fibers.

**Discrimination test**

Prior to our main study, we tested whether finches discriminate against cotton type and/or permethrin. We placed four dispensers at 100m intervals at the Charles Darwin Research Station. Each dispenser was loaded with 3g of each type of cotton and treatment: permethrin-treated processed and unprocessed cotton and water-treated processed and unprocessed cotton (Figure 1C). After 14 days, the cotton was weighed to the nearest 0.001g to determine how much of each type was removed from the

dispensers. Over the course of two weeks, there was no significant difference in the type of cotton birds removed from the dispensers: finches removed a mean (± SE) of 0.83 ± 0.46g processed permethrin cotton, 1.10 ± 0.64g unprocessed permethrin cotton, 0.90 ± 0.70g processed water cotton, and 0.95 ± 0.60g unprocessed water cotton (Kruskal Wallis, $H = 1.027$, $P = 0.80$).

**Distance traveled for cotton**

We also tested how far finches will transport cotton to their nests. We placed a dispenser with cotton in the field at a location away from our main study site. About four weeks later, we collected nests within 200 meters of the dispenser after birds were finished using the nests (Darwin's finches do not re-use the same nests [S2]). We dissected each nest to determine whether it contained any cotton. Two nests closest to the dispenser (7 and 17 meters) had cotton, but ten more distant nests (all >25 meters away) contained no cotton. Thus, we concluded that Darwin's finches at this site will transport cotton up to about 20 meters.

**Self-fumigation experiment**

Based on the preference test, 30 cotton dispensers were hung from trees 40 meters apart (approximately 2 meters above the ground) along two transects through our field site (Figure S1). Experimental dispensers contained processed cotton treated with a 1% permethrin solution; control dispersers contained unprocessed cotton treated with water. Thirty-five grams of experimental or control cotton were placed evenly over the bottom

three-quarters of each dispenser. The cotton was re-sprayed with permethrin or water every 8-10 days.

We searched for active Darwin's finch nests once a week for approximately 2 months after the dispensers were placed in the field. Once a nest was found, breeding activity (eggs or nestlings) was confirmed by checking the nest with a fiber optic camera (31mm in diameter, 36 mm in length; Sony®, Tokyo, Japan) attached to a 4m collapsible pole. During this visit, we also identified the species of Darwin's finch associated with each nest by briefly observing nest activity with binoculars from at least 5m away. Six of the experimental nests were built by *G. fortis*, five by *G. fuliginosa*, one by *C. parvulus*, and one by an unidentified finch species. Two of the control nests were built by *G. fortis*, one by *G. fuliginosa*, two by *C. parvulus*, one by *P. crassirostris*, and three by unidentified finch species.

Once nestlings had died or fledged, each nest was collected and sealed in a plastic bag. The nest was dissected within eight hours and any *P. downsi* larvae, pupae, and eclosed pupal cases were counted. First instar larvae can burrow subcutaneously into nestlings, making them impossible to quantify reliably [S3]. Therefore, as in previous studies [S3], total parasite abundance was the sum of all second and third instar larvae, pupae, and eclosed pupal cases in the nest material. Larvae and pupae removed from nests were reared to confirm their identification as *P. downsi* [S4].

All cotton was removed from nests and weighed to the nearest 0.001g. Non-cotton nest material was also weighed to the nearest 0.001g. The amount of cotton used in nests did not differ significantly by treatment. Thirteen experimental nests had a mean (± SE) of 2.12 ± 0.62g cotton; nine control nests had a mean of 1.04 ± 0.47g cotton

(Mann-Whitney test: $U = 40.00$, $P = 0.23$). The percent of nest material comprised of cotton did not differ significantly by treatment (experimental nests were 5.61 ± 1.87% cotton; control nests were 2.50 ± 1.23% cotton; Mann-Whitney test: $U = 39.00$, $P = 0.20$). Four nests did not contain any cotton; these nests had a mean of 48.25 ± 16.68 parasites.

**Effect of fumigation on fledging**

Active nests were visited every other day between 0600 and 1100h to record the number of eggs and nestlings present. Nests were randomly assigned to the experimental or control group. Experimental nests were sprayed with a 1% permethrin solution; control nests were sprayed with water. Nests were treated when the first nestling hatched, and again 4 days later. Nestlings, eggs and a thin layer of material from the bottom of the nest were removed before the nests were treated. Parents were quick to return to their nests following treatment, and there were no cases of nest abandonment due to treatment. Nestlings were marked shortly after hatching by coloring one toenail with a permanent marker. At ~8 days of age, nestlings were banded with a numbered monel metal band and three color bands. Banded nestlings were then re-sighted within seven days of leaving the nest to confirm fledging success. After the nest failed or all nestlings had fledged, the nest was collected and sealed in a plastic bag to quantify *P. downsi,* as described above. Three nests in the control treatment were overrun by fire ants (*Solenopsis geminata*) and therefore excluded from the analyses.

**Supplemental References**

S1. Foster, D. J., Podos, J., and Hendry, A. (2008). A geometric morphometric appraisal of beak shape in Darwin's finches. J. Evol. Biol. *21*, 263–75.

S2. Grant, P. (1999). Ecology and Evolution of Darwin's Finches (Princeton, New Jersey: Princeton University Press).

S3. Koop, J. A. H., Huber, S. K., Laverty, S. M., and Clayton, D. H. (2011). Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PLoS One *6*, e19706.

S4. Dodge, H. R., and Aitken, T. H. G. (1968). *Philornis* Flies from Trinidad (Diptera: Muscidae). J. Kansas Entomol. Soc. *41*, 134–154.

# APPENDIX A

# EXPERIMENTAL DEMONSTRATION OF A PARASITE-INDUCED IMMUNE RESPONSE IN WILD BIRDS: DARWIN'S FINCHES AND INTRODUCED NEST FLIES

Printed with permission from:
Koop, J.A.H., J.P. Owen, S.A. Knutie, M.A. Aguilar, D.H. Clayton. 2013. Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies. Ecology and Evolution 3: 2514-2523, doi: 10.1002/ece3.651.

Ecology and Evolution

Open Access

# Experimental demonstration of a parasite-induced immune response in wild birds: Darwin's finches and introduced nest flies

Jennifer A. H. Koop[1], Jeb P. Owen[2], Sarah A. Knutie[1], Maria A. Aguilar[1] & Dale H. Clayton[1]

[1]Department of Biology, University of Utah, Salt Lake City, Utah 84112
[2]Department of Entomology, Washington State University, Pullman, Washington 99164

**Keywords**
Antibody, defense, ecoimmunology, *Geospiza fortis*, invasive species, *Philornis downsi*.

**Correspondence**
Jennifer A. H. Koop, Department of Biology, University of Utah, Salt Lake City, UT 84112. Tel: +1 (520) 626-9315; Fax: +1 (520) 621-9190; E-mail: jenniferkoop@email.arizona.edu

**Present address**
Jennifer A. H. Koop Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721

**Funding Information**
The work was supported by National Science Foundation (DEB-0816877), a National Institutes of Health-Postdoctoral Fellowship (K12-GM000708), Sigma Xi Grants-in-aid of Research, a National Science Foundation Research Coordination Network in Ecoimmunology grant, the University of Utah Undergraduate Bioscience Research Program and the Undergraduate Research Opportunities Program.

Received: 21 February 2013; Revised: 10 May 2013; Accepted: 22 May 2013

doi: 10.1002/ece3.651

## Abstract

Ecological immunology aims to explain variation among hosts in the strength and efficacy of immunological defenses. However, a shortcoming has been the failure to link host immune responses to actual parasites under natural conditions. Here, we present one of the first experimental demonstrations of a parasite-induced immune response in a wild bird population. The recently introduced ectoparasitic nest fly *Philornis downsi* severely impacts the fitness of Darwin's finches and other land birds in the Galápagos Islands. An earlier study showed that female medium ground finches (*Geospiza fortis*) had *P. downsi*-binding antibodies correlating with presumed variation in fly exposure over time. In the current study, we experimentally manipulated fly abundance to test whether the fly does, in fact, cause changes in antibody levels. We manipulated *P. downsi* abundance in nests and quantified *P. downsi*-binding antibody levels of medium ground finch mothers, fathers, and nestlings. We also quantified host behaviors, such as preening, which can integrate with antibody-mediated defenses against ectoparasites. *Philornis downsi*-binding antibody levels were significantly higher among mothers at parasitized nests, compared to mothers at (fumigated) non-parasitized nests. Mothers with higher antibody levels tended to have fewer parasites in their nests, suggesting that antibodies play a role in defense against parasites. Mothers showed no behavioral changes that would enhance the effectiveness of the immune response. Neither adult males, nor nestlings, had *P. downsi*-induced immunological or behavioral responses that would enhance defense against flies. None of the parasitized nests fledged any offspring, despite the immune response by mothers. Thus, this study shows that, while the immune response of mothers appeared to be defensive, it was not sufficient to rescue current reproductive fitness. This study further shows the importance of testing the fitness consequences of immune defenses, rather than assuming that such responses increase host fitness.

## Introduction

Immune responses can protect hosts from the fitness costs of parasitism; however, the strength and effectiveness of immune-mediated defense varies among individuals. Variability has been linked to factors including, but not limited to, host reproductive condition (Horak et al. 1999; Ilmonen et al. 2002), stress (Lacoste et al. 2002), evolutionary history of exposure (Lee and Klasing 2004; Matson 2006; Bonneaud et al. 2012), and genetic factors (Beadell et al. 2007). The ability to identify underlying causes of variation is limited by the context in which studies are performed (Graham et al. 2011). A major challenge in ecological immunology has been drawing causal relationships between host immune responses and actual parasites, under natural conditions (Owen and Clayton 2007; Owen et al. 2010; Boughton et al. 2011). Studies that experimentally manipulate parasite abundance and test for parasite-induced host immune responses have the potential to be very informative; unfortunately, such studies are few in number (Buechler

et al. 2002; De Coster et al. 2010). In this article we report the results of one of the first such studies in a natural host–parasite system.

In the Galápagos Islands of Ecuador an introduced parasitic nest fly, *Philornis downsi*, has been implicated in recent population declines of several species of Darwin's finches (Dvorak et al. 2004, 2012; Wiedenfeld et al. 2007; O'Connor et al. 2010b,c). Adult flies, which are not parasitic, lay their eggs in the nests of finches (Couri and Carvalho 2003; Fessl et al. 2006), or in the nares (nostrils) of nestlings (Galligan and Kleindorfer 2009). Once the eggs hatch, the larvae live in the nest and feed on the blood of the nestling and adult female birds (Dudaniec et al. 2006; Huber et al. 2010). *Philornis downsi* is known to have a significant negative effect on the reproductive success of its hosts (reviewed in Koop et al. 2011).

A recent study by Huber et al. (2010) of medium ground finches (*Geospiza fortis*; Fig. 1) demonstrated increased levels of *P. downsi*-binding antibodies in birds during the nesting season, compared to birds sampled immediately prior to nesting. This increase in antibodies was observed in adult female birds, but not in adult males. Female finches incubate eggs and brood offspring, hypothetically increasing their exposure to *P. downsi* larvae in the nest. Male finches do not incubate eggs or brood nestlings. Although Huber et al. (2010) showed a correlation between nesting and increased antibody level, this correlation could be driven by other variables such as immune stimulation induced by breeding stress (Pruett 2003). An experimental manipulation of parasite abundance is needed to confirm the extent to which the immune response is actually caused by the parasite. To this end, we manipulated parasite abundance in nests to confirm that the observed changes in immune response are, in fact, induced by *P. downsi*, and are not the product of other temporal correlates.

We also monitored adult and nestling behavior with respect to *P. downsi* in the nest. Behavioral defense can be integrated with immune responses against ectoparasites (Lehane 2005). For example, host antibodies produced against salivary proteins of ectoparasites are known to promote pruritus (itching), alerting the host to the presence of parasites (Wikel 1996; Owen et al. 2009). Hosts that respond to the presence of biting insects with defensive behaviors, such as preening, are far more likely to injure, kill, or reduce the feeding time of the parasite (Dusbabek and Skarkovaspakova 1988; O'Connor et al. 2010a).

**Figure 1.** Photo of a female medium ground finch from El Garrapatero, Santa Cruz, Galapagos.

Yet another goal of this study was to investigate the role of immune responses in mitigating the fitness effects of *P. downsi*. Antibodies produced by hosts have the potential to act defensively against ectoparasites, like *P. downsi*, by facilitating the speed and intensity of inflammatory responses (Owen et al. 2010). Inflammation of the skin inhibits blood feeding by preventing parasites from reaching host blood vessels with their mouthparts. Ectoparasites feeding on inflamed tissues may also ingest defensive peptides, or lytic molecules produced by the host that impair parasite feeding and digestion (Owen et al. 2009). These components of the immune response can lead to dramatic reductions in the survival, development, and reproduction of parasites (Owen et al. 2009). Thus, we compared the level of immune response by finches to the abundance of *P. downsi* larvae in their nests.

Finally, we quantified host reproductive success to investigate potential fitness consequences of host immune responses. Immune responses, even those associated with negative consequences for parasites, do not necessarily lead to increases in host fitness (Sheldon and Verhulst 1996; Norris and Evans 2000). Mounting an immune response is energetically expensive and may involve tradeoffs with other fitness components, such as parental care or reproductive effort (Raberg et al. 2000). Thus, hosts mounting strong immune responses against a parasite may have reduced fitness if they are less able to care for their offspring. Conversely, the benefit of reducing parasite abundance may outweigh the costs of an immune response and lead to a net increase in host fitness. Host immune response and behavior, parasite abundance, and host fitness must be measured simultaneously to rigorously interpret the influence of host immune defense on host fitness (Graham et al. 2011).

## Material and Methods

### Site description and experimental design

The study was conducted during January–April 2010 on the island of Santa Cruz in the Galápagos Archipelago. Our field site, El Garrapatero, is a 1.5 × 1.5 km area in

the arid, coastal zone. Medium ground finches are abundant at El Garrapatero, where they nest primarily in giant prickly pear cacti (*Opuntia galapageia*) (Huber 2008). Clutch size ranges from 2 to 5 eggs, and females incubate for 10–14 days. Medium ground finch nestlings hatch asynchronously over a 2- to 4-day period. Nestlings spend 10–14 days in the nest, prior to fledging. In years of adequate food resources, medium ground finches can lay multiple clutches within a given breeding season, but they do not reuse the same nests (Grant 1999).

We experimentally manipulated the abundance of *P. downsi* in medium ground finch nests and monitored host fitness following treatment. Additional factors, such as poor weather conditions, may have contributed to variation in host fitness; however, these effects were expected to act equally on across treatments. Therefore, by using an experimental approach, we were able to isolate and quantify the effect of only *P. downsi* on host fitness. To manipulate parasite abundance, we sprayed a 1% aqueous permethrin solution (Permectrin™ II, KMG-Bernuth, Inc., Houston, TX) into experimental nests; control nests were sham-fumigated with water. Nests were treated when the first nestling hatched, and again 4 days later. At each time period, nestlings and eggs were briefly removed from the nest along with a thin layer of nest material from the bottom of the nest. The nest was sprayed with either permethrin or water using a generic spray bottle with a fine mist setting. The nest was allowed to dry for several minutes at which point the nest material, nestlings, and eggs were returned to the nest (typically within 10 min of removal). Parents were quick to return to nests following treatment, and there were no cases of nest abandonment due to treatment. If a single pair of birds nested more than once during the study period, the treatment was reversed between reproductive bouts.

Active nests were visited every other day between 0600 and 1100 h to record the number of eggs and nestlings present. We continued to monitor nests until all nestlings died or fledged. Nestlings were marked shortly after hatching by coloring one toenail with a permanent marker (Sharpie®, Newell Rubbermaid, Oak Brook, IL). At ~5 days of age, nestlings were banded with a numbered monel metal band and three color bands. Successful fledging was confirmed by observing and identifying birds after they left the nest using color band combinations (Koop et al. 2011). Once empty, nests were collected and placed in a sealed bag to quantify *P. downsi*.

## Parasite abundance

The 21 fumigated and 22 sham-fumigated nests were carefully dissected within 8 h of collection and any *P. downsi* larvae, pupae, and eclosed pupal cases were counted. First instar larvae can live subcutaneously in nestlings, making them impossible to quantify reliably. Therefore, total parasite abundance was the sum of all second and third instar larvae, pupae, and eclosed pupal cases found in the nest material or externally on nestlings. Larvae and pupae removed from nests were reared to confirm their identification as *P. downsi* (Dodge and Aitken 1968).

## Blood sampling

When the oldest nestling was ~5 days old, we used a mist net to capture the parent birds near the nest between 0600 and 0900 h. From each parent we collected a small blood sample (90 $\mu$L) in a microcapillary tube via brachial venipuncture ($n$ = 14 females and 10 males from fumigated nests, 15 females and 10 males from sham-fumigated nests). Adults were banded with a numbered monel metal band and three color bands before being released. We also collected a blood sample (30 $\mu$L) from each nestling when they were 5–6 days old via brachial venipuncture ($n$ = 59 nestlings from fumigated nests, 10 nestlings from sham-fumigated nests). Samples were stored on wet ice in the field, then in a −20°C freezer at a field station, and ultimately in a −80°C freezer for longer term storage after the field season.

## Immunology

We used enzyme-linked immunosorbent assays (ELISA) to detect the presence of *P. downsi*-binding antibodies in the plasma of finches. Our protocol was modified slightly from that of Huber et al. (2010). Briefly, 96-well plates were coated with 100 $\mu$L/well of *P. downsi* protein extract (capture antigen) diluted in carbonate coating buffer (0.05 mol/L, pH 9.6). Plates were incubated overnight at 4°C, then washed and coated with 200 $\mu$L/well of bovine serum albumin (BSA) blocking buffer and incubated for 30 min at room temperature on an orbital table. Between each of the following steps, plates were washed five times with a Tris-buffered saline wash solution, loaded as described, and incubated for 1 h on an orbital table at room temperature. Triplicate wells were loaded with 100 $\mu$L/well of individual finch plasma (diluted 1:500 in sample buffer). Plates were then loaded with 100 $\mu$L/well of Rabbit-αHOSP-IgY (primary detection antibody; diluted 1:10,000), followed by 100 $\mu$L/well of Goat-αRabbit-hrp (secondary detection antibody; diluted 1:20,000) (Bethyl Laboratories, Montgomery, TX). Finally, plates were loaded with 100 $\mu$L/well of peroxidase substrate (tetramethylbenzidine, TMB: Kirkegaard and Perry cat., Gaithersburg, MD, 50-77-03) and incubated for exactly 10 min. The reaction was stopped using 100 $\mu$L/well of 2 mol/L $H_2SO_4$. Optical density (OD) was measured

 

using a spectrophotometer (BioTek, PowerWave HT, Winooski, VT, 450-nanometer filter).

On each plate, a positive control of pooled plasma was used in triplicate to correct for inter-plate variation. In addition, each plate contained a nonspecific binding (NSB) sample in which capture antigen, detection antibody, and secondary detection antibody were added, but plasma was excluded. Finally, each plate included a blank sample in which only the detection antibody was added, but plasma and capture antigen were excluded. NSB absorbance values were subtracted from the mean OD value of each sample. Antibody levels were compared between fumigated and sham-fumigated nests for adult females, adult males, and nestlings using a two-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc tests ($\alpha < 0.05$). We performed a Pearson correlation analysis to examine the relationship between female OD values from sham-fumigated nests and parasite abundance.

### Behavior

We monitored parental and nestling activities using battery-powered Sony® (Tokyo, Japan) video camera systems. We placed small nest cameras (31 mm in diameter, 36 mm in length) in the tops of nests. The cameras were attached to small recording devices (PV700 Hi-res DVR, 8 × 12 × 3 cm, StuntCams, Grand Rapids, MI) hidden under brush. Behavior was recorded for ~3 h between 0600 and 1000 in haphazard subsamples of fumigated ($n = 9$) and sham-fumigated ($n = 9$) nests.

From the video recordings we quantified the amount of time males and females spent at the nest after nestlings hatched. We also quantified the following female behaviors, which were performed at the nest: feeding nestlings, nest sanitation, brooding nestlings, standing at the nest entrance, standing erect in the nest, self-preening, and allo-preening nestlings. All of these behaviors were mutually exclusive, with the exception of self-preening. Because females often preened while brooding nestlings, time spent self-preening was analyzed independently of other behaviors at the nest. All other behaviors are presented and analyzed as a proportion of time spent at the nest.

We also quantified the following male behaviors performed at the nest: feeding nestlings, nest sanitation, feeding the female, and standing at the nest entrance. Males do not brood nestlings. All of these male behaviors were mutually exclusive and are presented and analyzed as a proportion of the time observed at the nest. While at the nest, males were never observed preening themselves, or nestlings.

Time spent feeding nestlings was measured from the moment an adult began transferring food to a nestling until the adult's bill left contact with the last nestling. Nest sanitation was measured when an adult actively contacted the nest material with its bill (Christe et al. 1996). Brooding time was measured when a female was sitting in the nest in direct contact with nestlings. Males and females also spent time standing at the nest entrance, but only females spent time standing erect inside the nest. Males performed all behaviors from the nest entrance; they were never observed entering the nest.

We also quantified two nestling behaviors: self-preening, defined as the amount of time a nestling moved its bill in contact with its body, and agitation, defined as shaking, repositioning, or jumping in the nest. Parents often blocked the camera, interfering with our ability to see nestlings; therefore, we only quantified nestling behavior when parents were not present at the nest. Nestling behaviors are reported as the proportion of the time they were observed without the parents present.

All videos were watched and scored by a single observer (M. A. A.) who was blind to nest treatment. Videos were analyzed using VLC media player (VideoLAN) and Quicktime 10.0 (Apple, Inc., Cupertino, CA). Nestlings in the videos ranged in age from 2 to 6 days and clutch size ranged from 1 to 5 nestlings. A single day of video for each nest was paired between treatments, based on nestling age and clutch size, such that neither mean nestling age, nor mean clutch size, differed significantly between treatments. We quantified behaviors from 54 total hours of video, with an average of 3 h for each of the 18 nests (nine fumigated, nine sham-fumigated). Two nests, one from each treatment, had males that were never observed at the nest while videotaping occurred. Therefore, we report behavior for females from 18 nests and for males from only 16 nests.

We used Wilcoxon matched-pair tests to compare the mean (±SE) time spent performing behaviors between treatments. We used Chi-square tests to compare the allocation of time across all behaviors performed at the nest. All statistical analyses were performed using in Prism® v.5.0b (GraphPad Software, Inc., La Jolla, CA) or R v.2.12.2 (R Development Core Team, Vienna, Austria).

## Results

### Parasite abundance

The experimental treatment of nests with permethrin was effective in reducing parasite abundance. Sham-fumigated nests had a mean parasite abundance of 38.50 ± 5.13 *P. downsi*, compared to 0.23 ± 0.19 *P. downsi* in fumigated nests ($t = 7.40$, $P < 0.0001$; Fig. 2). Parasite abundance ranged from 5 to 79 parasites in sham-fumigated nests and from 0 to 4 parasites in fumigated nests. Nineteen fumigated nests were free of *P. downsi*; the remaining two

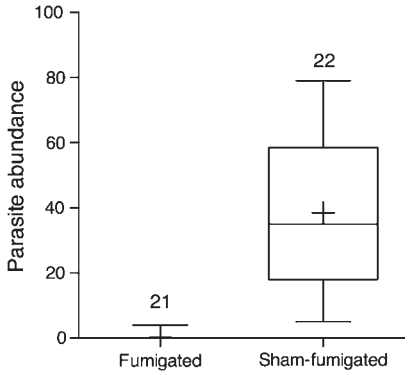


**Figure 2.** Box and whiskers plot of parasite abundance in fumigated and sham-fumigated nests. Boxes show the median, and the 25th% and 75th% for each treatment. Whiskers show the maximum and minimum values. The mean is indicated by a (+). The number of nests included in each treatment is shown above the bars.

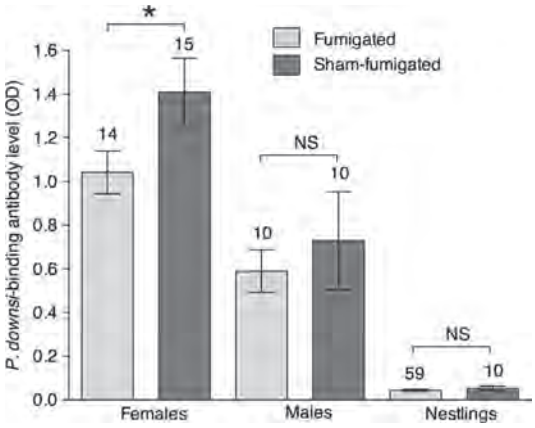


**Figure 3.** Mean (±SE) *Philornis downsi*-binding antibody response (optical density, OD) of adult females, adult males, and nestlings from fumigated and sham-fumigated nests. The number of individuals sampled is shown above each bar. Asterisk indicates a significant difference ($P < 0.05$) between treatments using Bonferroni post hoc comparisons (NS = nonsignificant).

nests, which experienced heavy rain soon after permethrin application, had very small numbers of *P. downsi* (one fly and four flies, respectively). *Philornis downsi* was found in all 22 sham-fumigated nests.

## Immunology

*Philornis downsi*-binding antibody responses differed significantly with family status, that is among adult females, adult males, and nestlings (two-way ANOVA; family status: $F_{2,\ 105} = 95.12$, $P < 0.001$; Fig. 3). There was also a significant effect of nest treatment (treatment: $F_{1,\ 105} = 5.18$, $P = 0.02$), but no significant interaction between treatment and family status (treatment × family status: $F_{2,\ 105} = 2.19$, $P = 0.12$). Bonferroni post hoc multiple comparisons showed that females in sham-fumigated nests had significantly greater *P. downsi*-binding antibody levels than females in fumigated nests ($t = 2.93$, $P < 0.05$). However, neither male nor nestling antibody levels differed significantly between treatments (males: $t = 1.02$, $P > 0.05$; nestlings: $t = 0.92$, $P > 0.05$). Hence, only females showed a significant, detectable antibody response to the experimental manipulation of *P. downsi* abundance in nests.

Among females from sham-fumigated nests ($n = 14$), *P. downsi*-binding antibody levels and parasite abundance were marginally correlated (Pearson correlation, $r = -0.51$, $P = 0.06$; Fig. 4). Females with greater *P. downsi*-binding antibody levels had fewer parasites in their nests.

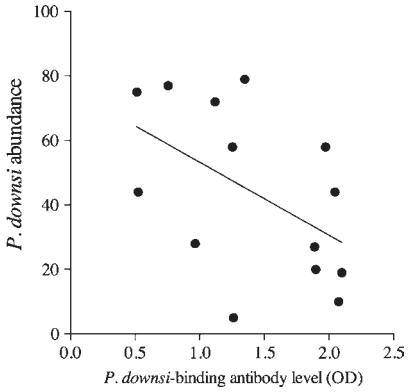


**Figure 4.** Relationship between adult female *Philornis downsi*-binding antibody level (optical density, OD) and *P. downsi* abundance in sham-fumigated nests. Each point represents a single female parent.

## Behavior

Females did not differ significantly in the amount of time they spent at fumigated and sham-fumigated nests ($n = 9$ females from fumigated nests, nine females from sham-fumigated nests; Wilcoxon -matched pairs, $W = -27.0$, $P = 0.13$); females from fumigated nests spent 44.1 ± 6.6% of their time at the nest, compared to 56.1 ± 9.1% for females at sham-fumigated nests. Females spent very little time self-preening at the nest, and there was no significant effect of treatment on self-preening ($W = 8.0$, $P = 0.59$); females in fumigated nests spent only 1.9 ± 1.4% of their time preening, compared to 1.4 ± 1.0% for females in sham-fumigated nests. Females spent <1% of their time allo-preening nestlings,

and there was no significant difference between treatments ($W = -10.0$, $P = 0.13$). Because preening was such an uncommon behavior, it was excluded from the additional comparisons of behavior below.

Females differed significantly in the time they devoted to different behaviors at fumigated versus sham-fumigated nests ($\chi^2 = 18.46$, df = 4, $P = 0.001$, Fig. 5A). The largest difference between treatments was in the time females spent brooding their offspring, versus standing erect in the nest. Females in fumigated nests spent 65 ± 8.7% of their time brooding, compared to 38 ± 9.1% by females in sham-fumigated nests. Females in fumigated nests spent 5.7 ± 1.1% of their time standing erect in the nest, compared to 21.5 ± 6.3% by females in sham-fumigated nests.

Males did not differ significantly between treatments in the amount of time they performed different behaviors while at the nest ($n = 8$ males from fumigated nests, eight males from sham-fumigated nests; $\chi^2 = 1.23$, $P = 0.75$; Fig. 5B). Overall, males spent very little time at the nest and there was no significant difference between treatments in nest attendance by males ($W = 29.0$, $P = 0.10$). Males in fumigated nests spent 3.2 ± 0.8% of their time at the nest, while males in sham-fumigated nests spent 1.6 ± 0.4% of their time at the nest.

Nestlings in fumigated nests tended to show less agitation (0.2 ± 0.1%) than nestlings in sham-fumigated nests (1.3 ± 0.7%), though the difference was not statistically significant ($W = -25.0$, $P = 0.09$). However, the agitation behavior of nestlings in sham-fumigated nests was significantly more variable than that of nestlings in fumigated nests (range within sham-fumigated nests, 0–5.27%; range within fumigated nests, 0–0.64%; *F*-test to compare variances, $F = 71.6$, df = 8.8, $P < 0.0001$). In short, the variation in agitation by nestlings in sham-fumigated nests was eightfold more than that of nestlings in fumigated nests. Nestlings were observed preening in only three nests (one sham-fumigated, two fumigated). Nestlings spent <1% of their time self-preening, which did not differ significantly between treatments ($W = -3$, $P = 0.50$).

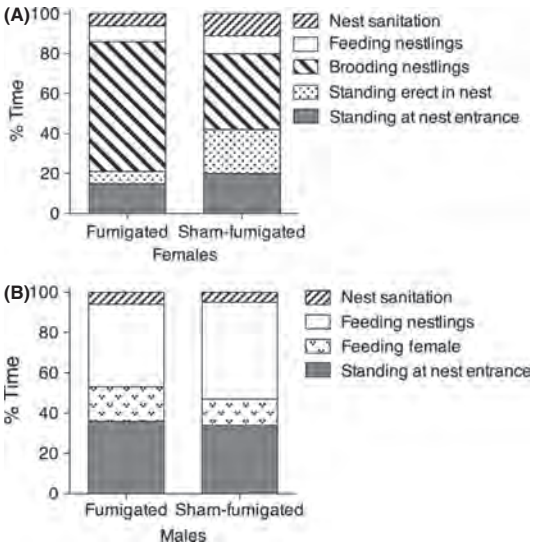


**Figure 5.** Mosaic plots of parental behaviors performed at the nest by (A) adult females and (B) adult males in fumigated and sham-fumigated nests ($N = 9$ nests per treatment for females; $N = 8$ nests per treatment for males).

### Fledging success

All 21 fumigated nests (100%) fledged at least one offspring, while none of the 22 sham-fumigated nests (0%) fledged any offspring (Fisher's Exact, $P < 0.0001$). Fifty-six of 74 (76%) nestlings fledged from fumigated nests, compared to 0 of 62 (0%) nestlings from sham-fumigated nests ($P < 0.0001$).

## Discussion

This study experimentally demonstrates a parasite-induced immune response in a wild bird population. Our results show a causal link between a biologically relevant host immune response and an actual parasite, under natural conditions. Adult female, but not male, medium ground finches produced a significant immunological (antibody-mediated) response to *P. downsi*. Our results show experimentally that *P. downsi* does, in fact, stimulate an immune response in adult females, consistent with the correlation reported by Huber et al. (2010). Furthermore, we show that females mounting stronger parasite-induced immune responses tend to have fewer parasites in their nests.

This study is one of the first demonstrations of an apparent effect of a parasite-induced immune response on parasite abundance in a wild bird. Work with other bird parasites has shown that antibody-mediated immune responses can increase the speed and intensity of the inflammatory response, preventing successful feeding of parasites and reducing parasite survival (Owen et al. 2009). Alternatively, the mechanism could be indirect; for example, female antibody responses may promote itching that alerts the host to biting insects (Wikel 1996; Owen et al. 2009). Females that respond with defensive behaviors, such as preening, could kill, injure, or remove parasites (Dusbabek and Skarkovaspakova 1988; O'Connor et al. 2010a). Further work is needed to explore additional variables that may be co-correlated with female immune response and parasite abundance.

A reduction in parasite burden is expected to benefit nestlings and thereby improve host reproductive success. However, within the parameters of this study, the observed decrease in parasite abundance did not help nestlings, as no nestlings fledged from any of the sham-fumigated nests. This result was surprising, given the results of a previous study performed in 2008 which showed that eradicating some, but not all, *P. downsi* from medium ground finch nests leads to increased fledging success (Koop et al. 2011). Koop et al. (2011) significantly reduced mean *P. downsi* abundance to ~21 parasites in nests treated with nest liners, compared to untreated nests, which had ~38 parasites per nest. This reduction was sufficient to increase fledgling success in lined nests, where 33% of nests fledged at least one offspring, compared to unlined nests, where only 4% of nests fledged any offspring. In this study, parasite abundance in sham-fumigated nests ranged from 5 to 79 *P. downsi* per nest (Fig. 2), yet no nestlings survived from nests in this treatment. The young age at which nestlings in this study died and the complete failure of nests even with low parasite abundance suggests that the impact of *P. downsi* on finches was unusually severe at our study site in 2010. One possible reason is that 2009 was a very dry year; annual rainfall in 2009 was 219 mm, compared to 503 mm in 2010 (Charles Darwin Foundation [2012], Meteorological Database). Dry years reduce overall seed availability, meaning that the seed bank in 2010 may have been depleted (Schluter 1982). Limited food resources are expected to negatively affect adult condition (Boag and Grant 1984), which may have placed additional stress on nestlings.

Furthermore, annual differences in rainfall may have contributed to changes in *P. downsi* virulence. Multiple *P. downsi* females can infest a single finch nest and female flies can mate with multiple males (Dudaniec et al. 2010). Thus, the relatedness between *P. downsi* larvae in a single finch nest has a relatively high degree of variability. Models of kin selection predict that when genetic relatedness of parasites is low, competition for within-host resources increases, leading to greater costs to the host (Frank 1994, 1996). While we did not collect data to directly test this idea, annual variation in climatic conditions may have altered the egg laying strategy of female flies, causing variation in parasite virulence between years. However, variation in parasite virulence could also be due to a number of other factors, such as host bird traits. Further investigation is needed to determine the role of biotic and abiotic factors on *P. downsi* virulence.

Independent of the effect on parasite abundance, female immune responses are thought to alter parental investment in current or future offspring (Raberg et al. 2000; Bonneaud et al. 2003). The ability of adult birds to perform parental behaviors can depend on the amount of energy invested (or not invested) in an immune response. Increases in nest sanitation and preening behaviors can serve to reduce parasite burden in the nest (Christe et al. 1996; Hurtrez-Bousses et al. 2000; Clayton et al. 2010). Parents can also alter the rate at which they feed nestlings in order to provide energetic compensation for the direct negative effects of parasitism (Tripet and Richner 1997; Hurtrez-Bousses et al. 1998). Alternatively, birds can abandon nests with parasites in favor of future reproductive efforts (Duffy 1983). O'Connor et al. (2010a) observed females of several finch species performing nest sanitation as well as allo-preening the feathers and nares of nestlings in nests with *P. downsi*. Interestingly, we observed almost no allo-preening; however, our observations were of younger nestlings (most of which were dead by 1 week of age). Our data show that while females did not abandon their parasitized nestlings or spend less time at the nest, they also did not significantly increase potentially beneficial behaviors, such as nest sanitation, or feeding nestlings. As in this study, O'Connor et al. (2010a) found no correlation between *P. downsi* intensity and parental feeding of nestlings.

Females in this study did, however, alter their brooding behavior; females in parasitized nests brooded significantly less and stood up more than females in fumigated nests. Whether this behavior was in response to agitated nestlings, or the parasites themselves, standing was probably an avoidance strategy for females (Hart 1990). Although this study shows that these responses were not sufficient to rescue current reproduction, further study is needed to determine whether female responses increase their ability to invest in future reproduction.

Young altricial nestlings are expected to serve as primary hosts for nest parasites because they lack the necessary motor skills to preen or stand. Furthermore, both the innate and acquired arms of the immune system are developing in nestlings, perhaps making them incapable of mounting a robust immune response to parasites (Palacios et al. 2009). We found no detectable difference in antibody levels of nestlings in fumigated and sham-fumigated nests. This result suggests that nestlings are not able to defend themselves immunologically against *P. downsi* in the nest. However, it should be noted that the rapid mortality of nestlings in sham-fumigated nests limited our sampling to young nestlings (~5 days of age). A recent study by King et al. (2010) found that nestlings of some species of birds can start producing parasite-induced antibodies endogenously within 3–6 days of age. Thus, quantification of antibodies from older nestlings (6–14 days old) may yield different results. Of course, the ability of nestlings to produce *P. downsi*-binding antibodies and immunologically defend themselves against nest parasites is dependent upon their survival to that time point.

Our results suggest that young nestlings are incapable of responding behaviorally to *P. downsi*. O'Connor et al. (2010a) observed medium ground finch nestlings preening themselves in a nest parasitized by *P. downsi*. The same nestlings were also observed trying to climb on top of one another, possibly to escape *P. downsi* larvae attempting to feed. Nestlings in this study were observed preening only rarely (<1% of time), and the behavior did not differ significantly between treatments. Nestlings from sham-fumigated nests tended to show more agitated behavior than those in fumigated nests. Periods of agitation included shaking and repositioning within the nest, but we did not observe nestlings standing on top of one another. Again, many of the nestlings observed by O'Connor et al. (2010a) were significantly older (>8 days of age), which may explain the differences in behavior between studies. Very young nestlings lack the necessary motor skills to preen themselves, or stand.

Studies that explore trade-offs between host immune response and life-history components often operate under the assumption that stronger immune responses are positively correlated with higher fitness (Norris and Evans 2000). This study demonstrates immunological activity of birds in response to a biologically relevant parasite. The data further suggest that stronger immune responses are defensive, because higher antibody levels are marginally correlated with lower parasite abundance. However, higher antibody levels did not result in higher reproductive success. This study provides a cautionary tale: even when stronger immune responses lead to lower parasite load, this does not necessarily result in higher host fitness. Our study underscores the importance of studying interactions between the host immune system, parasite load, and host fitness in order to derive robust conclusions regarding the functional significance of the immune system in an ecological context (Owen et al. 2010; Graham et al. 2011).

## Acknowledgments

We thank P. Espina and M. G. Clayton for help in the field or laboratory. We thank F. R. Adler, S. E. Bush, M. D. Dearing, S. K. Huber, and M. O. King for other forms of assistance with the research and preparation of this manuscript. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004) and by the Galápagos National Park (PC-04-10: #0054411). The study was supported by National Science Foundation grant DEB-0816877 to D. H. C., an National Institutes of Health-PERT Fellowship (K12-GM000708), Sigma Xi Grants-in-aid of Research, and an National Science Foundation Research Coordination Network grant in Ecoimmunology to J. A. H. K. M. A. A. was supported by the University of Utah Undergraduate Bioscience Research Program and Undergraduate Research Opportunities Program.

## Conflict of Interest

None declared.

### References

Beadell, J. S., C. Atkins, E. Cashion, M. Jonker, and R. C. Fleischer. 2007. Immunological change in a parasite-impoverished environment: divergent signals from four island taxa. PLoS One 2:e896. doi: //10.1371/journal/pone.0000896.

Boag, P. T., and P. R. Grant. 1984. Darwin's finches (Geospiza) on Isla Daphne Major, Galapagos: breeding and feeding ecology in a climatically variable environment. Ecol. Monogr. 54:463–489.

Bonneaud, C., J. Mazuc, G. Gonzalez, C. Haussey, O. Chastel, B. Faivre, et al. 2003. Assessing the cost of mounting an immune response. Am. Nat. 161:367–379.

Bonneaud, C., S. L. Balenger, G. E. Hill, and A. F. Russell. 2012. Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. Mol. Ecol. 21:4787–4796.

Boughton, R. K., G. Joop, and S. A. O. Armitage. 2011. Outdoor immunology: methodological considerations for ecologists. Funct. Ecol. 25:81–100.

Buechler, K., P. S. Fitze, B. Gottstein, A. Jacot, and H. Richner. 2002. Parasite-induced maternal response in a natural bird population. J. Anim. Ecol. 71:247–252.

Charles Darwin Foundation. 2012. CDF Meteorological Database. Available at http://www.darwinfoundation.org/datazone/climate/ (accessed 5 February 2012).

Christe, P., H. Richner, and A. Oppliger. 1996. Of great tits and fleas: sleep baby sleep. Anim. Behav. 52:1087–1092.

Clayton, D. H., J. A. H. Koop, C. W. Harbison, B. R. Moyer, and S. E. Bush. 2010. How birds combat ectoparasites. Open Ornithol. J. 3:41–71.

Couri, M. S., and C. J. B. Carvalho. 2003. Systematic relations among *Philornis* Meinert, *Passeromyia* Rodhain & Villeneuve and allied genera (Diptera, Muscidae). Braz. J. Biol. 63:223–232.

De Coster, G., L. De Neve, D. Martin-Galvez, L. Therry, and L. Lens. 2010. Variation in innate immunity in relation to ectoparasite load, age, and season: a field experiment in great tits (*Parus major*). J. Exp. Biol. 213:3012–3018.

Dodge, H. R., and T. H. G. Aitken. 1968. *Philornis* flies from Trinidad (Diptera: Muscidae). J. Kansas Entomol. Soc. 41:134–154.

Dudaniec, R. Y., S. Kleindorfer, and B. Fessl. 2006. Effects of the introduced ectoparasite *Philornis downsi* on haemoglobin level and nestling survival in Darwin's small ground finch (*Geospiza fuliginosa*). Austral Ecol. 31:88–94.

Dudaniec, R. Y., M. G. Gardner, and S. Kleindorfer. 2010. Offspring genetic structure reveals mating and nest infestation behaviour of an invasive parasitic fly (*Philornis downsi*) of Galapagos birds. Biol. Invasions 12:581–592.

Duffy, D. C. 1983. The ecology of tick parasitism on densely nesting Peruvian seabirds. Ecology 64:110–119.

Dusbabek, F., and V. Skarkovaspakova. 1988. Acquired-resistance of pigeons to Argas-polonicus larvae. Folia Parasitol. 35:77–84.

Dvorak, M., H. Vargas, B. Fessl, and S. Tebbich. 2004. On the verge of extinction: a survey of the mangrove finch *Cactospiza heliobates* and its habitat on the Galápagos Islands. Oryx 38:1–9.

Dvorak, M., B. Fessl, E. Nemeth, S. Kleindorfer, and S. Tebbich. 2012. Distribution and abundance of Darwin's finches and other land birds on Santa Cruz Island, Galapagos: evidence for declining populations. Oryx 46:78–86.

Fessl, B., B. J. Sinclair, and S. Kleindorfer. 2006. The life-cycle of *Philornis downsi* (Diptera: Muscidae) parasitizing Darwin's finches and its impacts on nestling survival. Parasitology 133:739–747.

Frank, S. A. 1994. Kin selection and virulence in the evolution of protocells and parasites. Proc. R. Soc. Lond. B Biol. Sci. 258:153–161.

Frank, S. A. 1996. Models of parasite virulence. Q. Rev. Biol. 71:37–78.

Galligan, T. H., and S. Kleindorfer. 2009. Naris and beak malformation caused by the parasitic fly, *Philornis downsi* (Diptera: Muscidae), in Darwin's small ground finch, *Geospiza fuliginosa* (Passeriformes: Emberizidae). Biol. J. Linn. Soc. 98:577–585.

Graham, A. L., D. M. Shuker, L. C. Pollitt, S. K. J. R. Auld, A. J. Wilson, and T. J. Little. 2011. Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct. Ecol. 25:5–17.

Grant, P. R. 1999. Ecology and evolution of Darwin's finches. Princeton Univ. Press, Princeton, NJ.

Hart, B. L. 1990. Behavioral adaptations to pathogens and parasites: five strategies. Neurosci. Biobehav. Rev. 14: 273–294.

Horak, P., L. Tegelmann, I. Ots, and A. P. Moller. 1999. Immune function and survival of great tit nestlings in relation to growth conditions. Oecologia 121:316–322.

Huber, S. K. 2008. Effects of the introduced parasite *Philornis downsi* on nestling growth and mortality in the medium ground finch (*Geospiza fortis*). Biol. Conserv. 141:601–609.

Huber, S. K., J. P. Owen, J. A. H. Koop, M. O. King, P. R. Grant, B. R. Grant, et al. 2010. Ecoimmunity in Darwin's finches: invasive parasites trigger acquired immunity in the medium ground finch (*Geospiza fortis*). PLoS One 5:e8605.

Hurtrez-Bousses, S., J. Blondel, P. Perret, J. Fabreguettes, and F. Renaud. 1998. Chick parasitism by blowflies affects feeding rates in a Mediterranean population of blue tits. Ecol. Lett. 1:17–20.

Hurtrez-Bousses, S., F. Renaud, J. Blondel, P. Perret, and M. J. Galan. 2000. Effects of ectoparasites of young on parents' behaviour in a Mediterranean population of blue tits. J. Avian Biol. 31:266–269.

Ilmonen, P., T. Taarna, and D. Hasselquist. 2002. Are incubation costs in female pied flycatchers expressed in humoral immune responsiveness or breeding success? Oecologia 130:199–204.

King, M. O., J. P. Owen, and H. G. Schwabl. 2010. Are maternal antibodies really that important? Patterns in the immunologic development of altricial passerine house sparrows (*Passer domesticus*). PLoS One 5:e9639. doi: 10.1371/journal.pone.0009639.

Koop, J. A. H., S. K. Huber, S. M. Laverty, and D. H. Clayton. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PLoS One 6:e19706. doi: 10.1371/journal.pone.0019706.

Lacoste, A., S. K. Malham, F. Gelebart, A. Cueff, and S. A. Poulet. 2002. Stress-induced immune changes in the oyster *Crassostrea gigas*. Dev. Comp. Immunol. 26:1–9.

Lee, K. A., and K. C. Klasing. 2004. A role for immunology in invasion biology. Trends Ecol. Evol. 19:523–529.

Lehane, M. 2005. The biology of blood-sucking insects. Cambridge Univ. Press, Cambridge.

Matson, K. D. 2006. Are there differences in immune function between continental and insular birds? Proc. Biol. Sci. 273:2267–2274.

Norris, K., and M. R. Evans. 2000. Ecological immunology: life history trade-offs and immune defense in birds. Behav. Ecol. 11:19–26.

O'Connor, J., J. Robertson, and S. Kleindorfer. 2010a. Video analysis of host-parasite interactions in nests of Darwin's finches. Oryx 44:588–594.

O'Connor, J., F. J. Sulloway, and S. Kleindorfer. 2010b. Avian population survey in the Floreana highlands: is Darwin's medium tree finch declining in remnant patches of *Scalesia* forest? Bird Conserv. Int. 20:343–353.

O'Connor, J., F. J. Sulloway, J. Robertson, and S. Kleindorfer. 2010c. *Philornis downsi* parasitism is the primary cause of nestling mortality in the critically endangered Darwin's medium tree finch (*Camarhynchus pauper*). Biodivers. Conserv. 19:853–866.

Owen, J. P., and D. H. Clayton. 2007. Where are the parasites in the PHA response? Trends Ecol. Evol. 22: 228–229.

Owen, J. P., M. Delany, C. Cardona, A. Bickford, and B. Mullens. 2009. Host inflammatory response governs fitness in an avian ectoparasite, the northern fowl mite (*Ornithonyssus sylviarum*). Int. J. Parasitol. 39:789–799.

Owen, J. P., A. C. Nelson, and D. H. Clayton. 2010. Ecological immunology of bird-ectoparasite systems. Trends Parasitol. 26:530–539.

 

Palacios, M. G., J. E. Cunnick, D. Vleck, and C. M. Vleck. 2009. Ontogeny of innate and adaptive immune defense components in free-living tree swallows, *Tachycineta bicolor*. Dev. Comp. Immunol. 33:456–463.

Pruett, S. B. 2003. Stress and the immune system. Pathophysiology 9:133–153.

Raberg, L., J. A. Nilsson, P. Ilmonen, M. Stjernman, and D. Hasselquist. 2000. The cost of an immune response: vaccination reduces parental effort. Ecol. Lett. 3:382–386.

Schluter, D. 1982. Seed and patch selection by Galapagos ground finches – relation to foraging efficiency and food-supply. Ecology 63:1106–1120.

Sheldon, B. C., and S. Verhulst. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11:317–321.

Tripet, F., and H. Richner. 1997. Host responses to ectoparasites: food compensation by parent blue tits. Oikos 78:557–561.

Wiedenfeld, D. A., G. A. Jimenez, B. Fessl, S. Kleindorfer, and J. C. Valarezo. 2007. Distribution of the introduced parasitic fly *Philornis downsi* (Diptera, Muscidae) in the Galapagos Islands. Pac. Conserv. Biol. 13:14–19.

Wikel, S. K. 1996. The immunology of host-ectoparasitic arthropod relationships. Cab International, Oxon, U.K.

# APPENDIX B

# EPIGENETICS AND THE EVOLUTION OF DARWIN'S FINCHES

Printed with permission from:
Skinner, M.K., C. Guerrero-Bosagna, M.M. Haque, J.A.H. Koop, S.A. Knutie, and D.H. Clayton. 2013. Epigenetics and the evolution of Darwin's finches. Genome Biology and Evolution 6: 1972-1989, doi: 10.1093/gbe/evu158.

GENOME BIOLOGY AND EVOLUTION
SMBE

GBE

# Epigenetics and the Evolution of Darwin's Finches

Michael K. Skinner[1,*], Carlos Gurerrero-Bosagna[1,3], M. Muksitul Haque[1], Eric E. Nilsson[1], Jennifer A.H. Koop[2,4], Sarah A. Knutie[2], and Dale H. Clayton[2]

[1]Center for Reproductive Biology, School of Biological Sciences, Washington State University

[2]Department of Biology, University of Utah

[3]Present address: Department of Physics, Biology and Chemistry (IFM), Linköping University, Sweden

[4]Present address: Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ

*Corresponding author: E-mail: skinner@wsu.edu.

**Accepted:** July 18, 2014

**Data deposition:** All DMR and CNV genomic data obtained in this study have been deposited in the NCBI public GEO database under the accession (GEO #: GSE58334).

## Abstract

The prevailing theory for the molecular basis of evolution involves genetic mutations that ultimately generate the heritable phenotypic variation on which natural selection acts. However, epigenetic transgenerational inheritance of phenotypic variation may also play an important role in evolutionary change. A growing number of studies have demonstrated the presence of epigenetic inheritance in a variety of different organisms that can persist for hundreds of generations. The possibility that epigenetic changes can accumulate over longer periods of evolutionary time has seldom been tested empirically. This study was designed to compare epigenetic changes among several closely related species of Darwin's finches, a well-known example of adaptive radiation. Erythrocyte DNA was obtained from five species of sympatric Darwin's finches that vary in phylogenetic relatedness. Genome-wide alterations in genetic mutations using copy number variation (CNV) were compared with epigenetic alterations associated with differential DNA methylation regions (epimutations). Epimutations were more common than genetic CNV mutations among the five species; furthermore, the number of epimutations increased monotonically with phylogenetic distance. Interestingly, the number of genetic CNV mutations did not consistently increase with phylogenetic distance. The number, chromosomal locations, regional clustering, and lack of overlap of epimutations and genetic mutations suggest that epigenetic changes are distinct and that they correlate with the evolutionary history of Darwin's finches. The potential functional significance of the epimutations was explored by comparing their locations on the genome to the location of evolutionarily important genes and cellular pathways in birds. Specific epimutations were associated with genes related to the bone morphogenic protein, toll receptor, and melanogenesis signaling pathways. Species-specific epimutations were significantly overrepresented in these pathways. As environmental factors are known to result in heritable changes in the epigenome, it is possible that epigenetic changes contribute to the molecular basis of the evolution of Darwin's finches.

**Key words:** epimutations, DNA methylation, copy number variation, phylogeny, adaptive radiation, BMP, toll, melanogenesis.

## Introduction

Epigenetic change has been postulated to play a role in the ecology and evolution of natural populations (Richards et al. 2010; Holeski et al. 2012; Liebl et al. 2013). Epigenetic changes are broadly defined as "molecular processes around DNA that regulate genome activity independent of DNA sequence and are mitotically stable" (Skinner et al. 2010). Some epigenetic processes are also meiotically stable and are transmitted through the germline (Anway et al. 2005; Jirtle and Skinner 2007). These epigenetic mechanisms, such as DNA methylation, can become programmed (e.g., imprinted) and inherited over generations with potential evolutionary impacts. Environmental factors have been shown to promote the epigenetic transgenerational inheritance of phenotypic variants (Skinner et al. 2010). In recent years, the importance of environmental cues in the induction of such variation has been widely acknowledged (Bonduriansky 2012). Thus, like genetic change (Greenspan 2009), epigenetic change may also play an important role in evolution (Guerrero-Bosagna et al. 2005; Day and Bonduriansky 2011; Geoghegan and Spencer 2012, 2013a, 2013b, 2013c; Klironomos et al. 2013).

In order for inherited epigenetic changes to play a significant role in microevolution, they must persist for tens of generations, or longer (Slatkin 2009). It is conceivable that epigenetic changes may also accumulate over longer periods of evolutionary time, contributing to processes such as adaptive radiation (Rebollo et al. 2010; Flatscher et al. 2012). This hypothesis assumes that epigenetic changes persist over thousands of generations. An initial step in testing this hypothesis would be to compare epigenetic differences among closely related species, and whether such changes accumulate over short spans of macroevolutionary time. For example, do epigenetic changes accumulate with phylogenetic distance? Addressing this question was the primary goal of this study.

The study was designed to explore the relationship between epigenetic changes and the evolutionary history of several species of Darwin's finches in the Galapagos Islands. This group of birds has been central to work on a variety of important topics in evolutionary biology, including adaptive radiation, character displacement, rapid evolution, hybridization between species, evolutionary developmental mechanisms, and the effect of invasive pathogens and parasites (Grant and Grant 2008; Huber et al. 2010; Donohue 2011). The adaptive radiation of Darwin's finches over a period of 2–3 Myr resulted in 14 extant species that fill distinct ecological niches. These species show striking variation in body size and the size and shape of their beaks (Grant and Grant 2008). Darwin's finches were selected for study because they are a well-studied example of the evolution of closely related species into different ecological niches (Grant and Grant 2008; Donohue 2011).

Natural selection is a process in which environmental factors influence the survival and reproductive success of individuals bearing different phenotypes. Only selection on phenotypic traits with a heritable basis can lead to evolutionary change (Endler 1986). Observations indicate that epigenetic mechanisms have a role in influencing genomic variability (Huttley 2004; Ying and Huttley 2011). As epigenetic changes are also influenced by environmental factors, and can be heritable across generations (Skinner et al. 2010), they provide another molecular mechanism that can influence evolutionary change. Although Lamarck (1802) proposed that environmental factors can influence inheritance directly, his mechanism has not been widely recognized as a component of modern evolutionary theory (Day and Bonduriansky 2011). Recent work in epigenetics shows that epigenetic changes can, in fact, increase the heritable phenotypic variation available to natural selection (Holeski et al. 2012; Liebl et al. 2013). Thus, epigenetics appears to provide a molecular mechanism that can increase phenotypic variation on which selection acts (Skinner 2011). The integration of genetic and epigenetic mechanisms has the potential to significantly expand our understanding of the origins of phenotypic variation and how environment can influence evolution.

For example, Crews et al. (2007) investigated the ability of an environmental factor (toxicant) to promote the epigenetic transgenerational inheritance of alterations in the mate preferences of rats, with consequences for sexual selection. An F0 generation gestating female rat was exposed to the agricultural fungicide vinclozolin transiently. A dramatic alteration in the mate preferences of the F3 generation was observed (Crews et al. 2007) along with epigenetic alterations (termed epimutations) in the germline (sperm) (Guerrero-Bosagna et al. 2010). Transgenerational transcriptome changes in brain regions correlated with these alterations in mate preference behavior were also observed (Skinner et al. 2008, 2014). Thus, an environmental factor that altered mate preference was found to promote a transgenerational alteration in the sperm epigenome in an imprinted-like manner that was inherited for multiple generations (Crews et al. 2007; Skinner et al. 2010). Studies such as these suggest that environmental epigenetics may play a role in evolutionary changes through processes, such as sexual selection.

Recent reviews suggest a pervasive role for epigenetics in evolution (Rebollo et al. 2010; Day and Bonduriansky 2011; Kuzawa and Thayer 2011; Flatscher et al. 2012; Klironomos et al. 2013). The primary goal of this study was to test whether epigenetic changes accumulate over the long periods of evolutionary time required for speciation with adaptive radiation. Genome wide analyses were used to investigate changes in genetic and epigenetic variation among five species of Darwin's finches. The measure of genetic variation was copy number variation (CNV), which has been shown to provide useful and stable genetic markers with potentially more phenotypic functional links than point mutations such as single nucleotide polymorphisms (SNPs) (Lupski 2007; Sudmant et al. 2013). CNVs involve an increase or decrease in the number of copies of a repeat element at a specific genomic location. Recently, CNV changes in primates and other species have been shown to be very useful genetic measures for comparing evolutionary events (Nozawa et al. 2007; Gazave et al. 2011; Poptsova et al. 2013). CNV changes are involved in gene duplication and deletion phenomena, as well as repeat element phenomenon such as translocation events and can be influenced by DNA methylation (Skinner et al. 2010; Macia et al. 2011; Tang et al. 2012). The measure of epigenetic variation used was differential DNA methylation sites, which are known to be stable and heritable (Skinner et al. 2010). Comparing data for both genetic mutations (i.e., CNV) and epimutations (i.e., DNA methylation) allowed the relative magnitudes of these sources of variation to be compared across the five species included in the study.

## Materials and Methods

### Finch Field Work and Collection of Blood

Blood samples were collected from birds captured January–April 2009 at El Garrapatero, a lowland arid site on Santa Cruz Island, Galapagos Archipelago, Ecuador (Koop et al. 2011).

Genome Biology and Evolution

SMBE

Birds were captured with mist nests and banded with numbered Monel bands to track recaptures. Birds were identified, aged, and sexed using size and plumage characteristics. A small blood sample (90 μl) from each bird was collected in a microcapillary tube through brachial venipuncture. Samples were stored on wet ice in the field, then erythrocytes purified by centrifugation and cells stored in a −20 °C freezer at a field station. Following the field season, samples were placed in a −80 °C freezer for longer term storage. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #07-08004) and by the Galápagos National Park (PC-04-10: #0054411).

### DNA Processing

Erythrocyte DNA was isolated with DNAeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and then stored at −80 °C prior to analysis. DNA was sonicated following a previously described protocol (without protease inhibitors) (Tateno et al. 2000) and then purified using a series of washes and centrifugations (Ward et al. 1999) from variable number of animals per species analyzed. The same concentrations of DNA from individual blood samples were then used to produce pools of DNA material. Two DNA pools were produced in total per species, each one containing the same amount of DNA from different animals. The number of individuals used per pool is shown in supplementary table S6, Supplementary Material online. These DNA pools were then used for chromosomal genomic hybridization (CGH) arrays or chromatin immunoprecipitation of methylated DNA fragments (MeDIP).

### CNV Analysis

The array used for the CNV analysis was a CGH custom design by Roche Nimblegen that consisted of a whole-genome tiling array of zebra finch (*Taeniopygia guttata*) with 720,000 probes per array. The probe size ranged from 50 to 75 mer in length with median probe spacing of 1,395 bp. Two different comparative (CNV vs. CNV) hybridization experiments were performed (two subarrays) for each species in query (*Geospiza fuliginosa* [FUL], *G. scandens* [SCA], *Camarhynchus parvulus* [PAR], and *Platyspiza crassirostris* [CRA]) versus control *G. fortis* (FOR), with each subarray including hybridizations from DNA pools from these different species. Two DNA pools were built for each species (supplementary table S6, Supplementary Material online). For one subarray of each species, DNA samples from the experimental groups were labeled with Cy5 and DNA samples from the control lineage were labeled with Cy3. For the other subarray of each species, a dye swap was performed so that DNA samples from the experimental groups were labeled with Cy3 and DNA samples from the control lineage were labeled with Cy5.

For the CNV experiment raw data from the Cy3 and Cy5 channels were imported into R (R Development Core Team 2010), checked for quality, and converted to *MA* values ($M = \text{Cy5} - \text{Cy3}$; $A = [\text{Cy5} + \text{Cy3}]/2$). Within array and between array normalizations were performed as previously described (Manikkam et al. 2012). Following normalization, the average value of each probe was calculated and three different CNV algorithms were used on each of these probes including circular binary segmentation from the DNA copy (Olshen et al. 2004), CGHseg (Picard et al. 2005) and cghFlasso (Tibshirani and Wang 2008). These three algorithms were used with the default parameters. The average values from the output of these algorithms were obtained. A threshold of 0.04 as a cutoff was used on the summary (average of the log-ratio from the three algorithms) where gains are probes above the positive threshold and losses are probes below the negative threshold. Consecutive probes (≥3) of gains and losses were used to identify separate CNV regions. A cutoff of three-probe minimum was used and those regions were considered a valid CNV. The statistically significant CNVs were identified and *P* values associated with each region presented. A cutoff of $P < 10^{-5}$ was used to select the final regions of gains and losses.

### Differential DNA Methylation Regions Analysis

MeDIP was performed as previously described (Guerrero-Bosagna et al. 2010) as follows: 6 μg of genomic DNA was subjected to series of three 20-pulse sonications at 20% amplitude and the appropriate fragment size (200–1,000 ng) was verified through 2% agarose gels; the sonicated genomic DNA was resuspended in 350 μl TE buffer and denatured for 10 min at 95 °C and then immediately placed on ice for 5 min; 100 μl of 5× IP buffer (50 mM Na-phosphate pH 7, 700 mM NaCl (PBS), 0.25% Triton X-100) was added to the sonicated and denatured DNA. An overnight incubation of the DNA was performed with 5 μg of antibody anti-5-methylCytidine monoclonal from Diagenode (Denville, NJ) at 4 °C on a rotating platform. Protein A/G beads from Santa Cruz were prewashed on PBS–BSA (bovine serum albumin) 0.1% and resuspended in 40 μl 1× IP (immunoprecipitation) buffer. Beads were then added to the DNA-antibody complex and incubated 2 h at 4 °C on a rotating platform. Beads bound to DNA-antibody complex were washed three times with 1 ml 1× IP buffer; washes included incubation for 5 min at 4 °C on a rotating platform and then centrifugation at 6,000 rpm for 2 min. Beads DNA-antibody complex were then resuspended in 250 μl digestion buffer (50 mM Tris–HCl pH 8, 10 mM ethylenediaminetetraacetic acid, 0.5% SDS (sodium dodecyl sulfate) and 3.5 μl of proteinase K (20 mg/ml) was added to each sample and then incubated overnight at 55 °C on a rotating platform. DNA purification was performed first with phenol and then with chloroform:isoamyl alcohol. Two washes were then performed with 70% ethanol, 1 M NaCl, and glycogen. MeDIP-selected DNA was then resuspended in 30 μl TE buffer.

The array used for the differential methylation analysis was a DNA-methylated custom array by Roche Nimblegen that

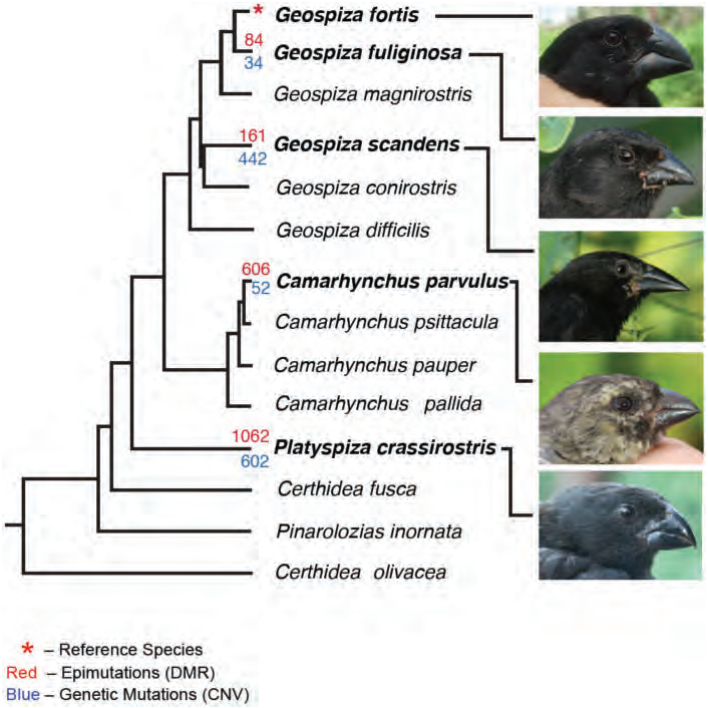


**Fig. 1.**—Number of epimutations and genetic mutations in relation to the phylogenetic relationships of five species of Darwin's finches. Photographs (by J.A.H.K. or S.A.K.) show variation in bill size and shape. Numbers on branches are the number of differences (three or more probes; table 1) in epimutations (DMR; in red) .and genetic mutations (CNV; in blue) for each of four species, compared with a single reference species FOR (asterisk). The phylogram is based on allele length variation at 16 polymorphic microsatellite loci (from Petren et al. 1999). The topology of the tree is similar to that proposed by Lack (1947) on the basis of morphological traits.

consisted of a whole-genome tiling array of zebra finch (*Taeniopygia guttata*) made of four 2.1M and one 3x720k array with 8,539,570 probes per array. Probe sizes were 50–75 mer in length and median probe spacing was 200 bp. Two different comparative (MeDIP vs. MeDIP) hybridization experiments were performed (two subarrays) for each experimental species (FUL, SCA, PAR, CRA) versus control FOR, with each subarray including hybridizations from MeDIP DNA from DNA pools from these different species (supplementary table S6, Supplementary Material online). For one subarray of each species, MeDIP DNA samples from the experimental groups were labeled with Cy5 and MeDIP DNA samples from the control lineage were labeled with Cy3. For the other subarray of each species, a dye swap was performed so that MeDIP DNA samples from the experimental groups were labeled with Cy3 and MeDIP DNA samples from the control lineage were labeled with Cy5.

For each comparative hybridization experiment, raw data from both the Cy3 and Cy5 channels were imported into R, checked for quality, and converted into *MA* values. The normalization procedure is as previously described (Guerrero-Bosagna et al. 2010). Following normalization each adjacent $\geq$3 probe set value represents the median intensity difference between FUL, SCA, PAR and CRA and control FOR of a 600-bp window. Significance was assigned to probe differences between experimental species samples and reference FOR samples by calculating the median value of the intensity differences as compared with a normal distribution scaled to the experimental mean and standard deviation of the normalized data. A *Z* score and *P* value were computed for each probe from that distribution. The statistically significant differential DNA methylation regions (DMR) were identified and *P* values associated with each region represented, as previously described (Guerrero-Bosagna et al. 2010).

A

| Differential DNA Methylation Regions (DMR) (Epimutations) | | | | | | | |
|---|---|---|---|---|---|---|---|
| All probes ($p<10^{-5}$) | | | | 3 or more probes ($p<10^{-5}$) | | | |
| | (Up) | (Down) | Total | | (Up) | (Down) | Total |
| FUL | 116 | 398 | 514 | FUL | 76 | 8 | 84 |
| SCA | 211 | 679 | 890 | SCA | 17 | 144 | 161 |
| PAR | 191 | 1438 | 1629 | PAR | 28 | 578 | 606 |
| CRA | 361 | 2406 | 2767 | CRA | 61 | 1001 | 1062 |
| | | | | | | | |
| Total | Up | Down | Total Sites | Total | Up | Down | Total Sites |
| | 879 | 4921 | 5800 | | 182 | 1731 | 1913 |

| Copy Number Variation (CNV) | | | | | | | |
|---|---|---|---|---|---|---|---|
| All probes ($p<10^{-5}$) | | | | 3 or more probes ($p<10^{-5}$) | | | |
| | Gains | Loss | Total | | Gains | Loss | Total |
| FUL | 59 | 12 | 71 | FUL | 28 | 6 | 34 |
| SCA | 567 | 22 | 589 | SCA | 440 | 2 | 442 |
| PAR | 78 | 217 | 295 | PAR | 15 | 37 | 52 |
| CRA | 621 | 194 | 815 | CRA | 541 | 61 | 602 |
| | | | | | | | |
| Total | Gains | Loss | Total Sites | Total | Gains | Loss | Total Sites |
| | 1325 | 445 | 1770 | | 1024 | 106 | 1130 |

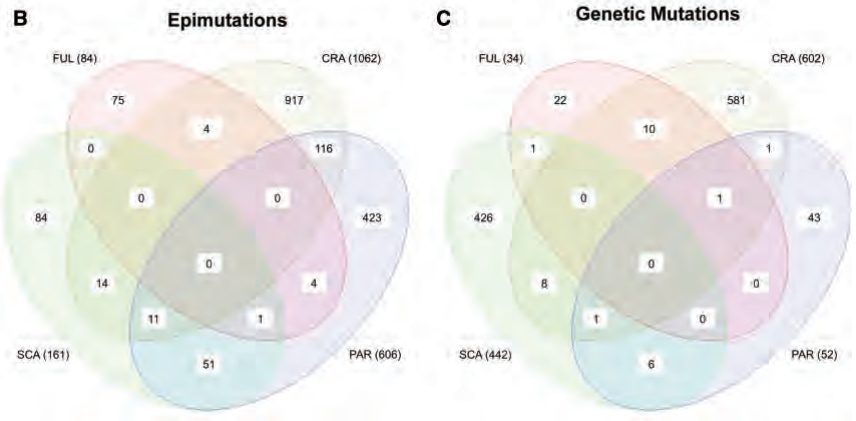


**Fig. 2.**—Number of epimutations and genetic mutations associated with Darwin's finches. The number of differential DMR epimutations and CNV genetic mutations (*A*). DMR and CNV that differ significantly ($P < 10^{-5}$) from the reference species (FOR) are presented for all oligonucleotide probes, compared with peaks of three or more adjacent probes. The epimutations with an increase (Up) or decrease (Down) in DNA methylation are indicated. Those genetic mutations with an increase (Gain) or decrease (Loss) in CNV are indicated. Venn diagrams for epimutations (*B*) and genetic mutations (*C*) show overlaps between epimutations (DMR) and genetic mutations (CNV) among species. The species and total number of sites compared are listed on the outside of each colored elliptical.

## Additional Bioinformatics and Statistics

The July 2008 assembly of the zebra finch genome (taeGut1, WUSTL v3.2.4) produced by the Genome Sequencing Center at the Washington University in St Louis (WUSTL) School of Medicine was retrieved (WUSTL 2008). A seed file was constructed and a BSgenome package was forged for using the Finch DNA sequence in the R code (Herve Pages BSgenome: Infrastructure for Biostrings-based genome data packages. R

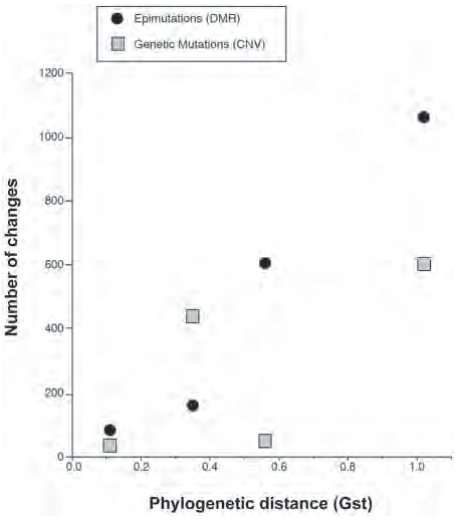


**Fig. 3.**—Phylogenetic distance is correlated with epigenetic changes, but not genetic changes. Branch lengths in figure 1 were used as measures of phylogenetic distance. The number of epimutations increased with phylogenetic distance (Spearman Rho = 1.0, $P < 0.0001$). In contrast, the number of genetic mutations did not increase with phylogenetic distance (Spearman Rho = 0.8, $P = 0.2$).

package version 1.24.0). This sequence was used to design the custom tiling arrays and to perform the bioinformatics.

The chromosomal location of CNV and DMR clusters used an R-code developed to find chromosomal locations of clusters (Skinner et al. 2012). A 2-Mb sliding window with 50,000 base intervals was used to find the associated CNV and DMR in each window. A *Z*-test statistical analysis with $P < 0.05$ was used on these windows to find the ones with overrepresented CNV and DMR were merged together to form clusters. A typical cluster region averaged approximately 3 Mb in size.

The DMR and CNV association with specific zebra finch genes and genome locations used the Gene NCBI database for zebra finch gene locations and correlated the epimutations associated (overlapped) with the genes. The three adjacent probes constituted approximately a 200-bp homology search. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway associations were identified as previously described (Skinner et al. 2012). Statistically significant overrepresentation uses a Fisher's exact analysis.

Spearman Rank correlation coefficients were used to test for a relationship between phylogenetic distance and epigenetic and genetic changes (Whitlock and Schluter 2009).

## Results

Phylogenetic relationships of the five finch species in this study are shown in figure 1. The taxa chosen for this study included: Two species of ground finches, FOR and FUL, which have crushing beaks with relatively deep bases; the cactus finch SCA, which has a long thin beak used for probing flowers; the small tree finch PAR, which has curved mandibles used for applying force at the tips; and the vegetarian finch CRA, which has a relatively short stubby bill used for crushing food along its entire length (Grant and Grant 2008; Donohue 2011; Rands et al. 2013). FOR was selected as a reference species for comparing genetic and epigenetic alterations among the remaining four species. Branch lengths in figure 1 were used as measures of phylogenetic distance.

The experimental design used purified erythrocytes from the different species. Although DNA sequences are the same for all cell types of an organism, the epigenome is distinct for each cell type, providing a molecular mechanism for the genome activity and functions that differ among different cell types (Skinner et al. 2010). Therefore, to investigate the overall epigenome requires a purified cell type. As birds have erythrocytes (red blood cells) that contain nuclei, samples of purified erythrocytes were collected from each of the Darwin's finch species to obtain DNA for molecular analysis.

The epigenetic alterations termed epimutations were assessed through the identification of differential DMR. The DMR were identified with the use of MeDIP with a methyl cytosine antibody, followed by a genome wide tiling array (Chip) for an MeDIP-Chip protocol (Guerrero-Bosagna et al. 2010). Although other epigenetic processes such as histone modifications, chromatin structure, and noncoding RNA are also important, DNA methylation is the best known epigenetic process associated with germline-mediated heritability and environmental manipulations (Skinner et al. 2010). Genetic variation was assessed using CNVs (i.e., amplifications and deletions of repeat elements) in the DNA using a CGH protocol (Pinkel and Albertson 2005; Gazave et al. 2011).

The reference genome used for the analysis was that of the zebra finch (*Taeniopygia guttata*) (Clayton et al. 2009), which had a preliminary estimate of greater than 83% similarity with a partial shotgun sequence of a Darwin's finch genome (Rands et al. 2013). This study actually suggests a much higher degree of identity. The zebra finch genome was tiled in a genome wide array with a 200-bp resolution and for a CGH array with a 1,500-bp resolution. These arrays were used in a competitive hybridization protocol between FOR (reference species) and the other four species (Guerrero-Bosagna et al. 2010). Differential hybridization using two different fluorescent DNA labeling tags identified the CNV with CGH using genomic DNA and the epimutation DMR with a MeDIP-Chip protocol. A statistical significance threshold of $P < 10^{-5}$ was set for the CNV or epimutation to be identified as a gain or loss compared with the reference species (fig. 2 and supplementary tables S1 and S2, Supplementary Material online). The data for all probes (oligonucleotides on the arrays) are presented. However, the criteria used to identify the CNV and DMR required the involvement of three or more adjacent

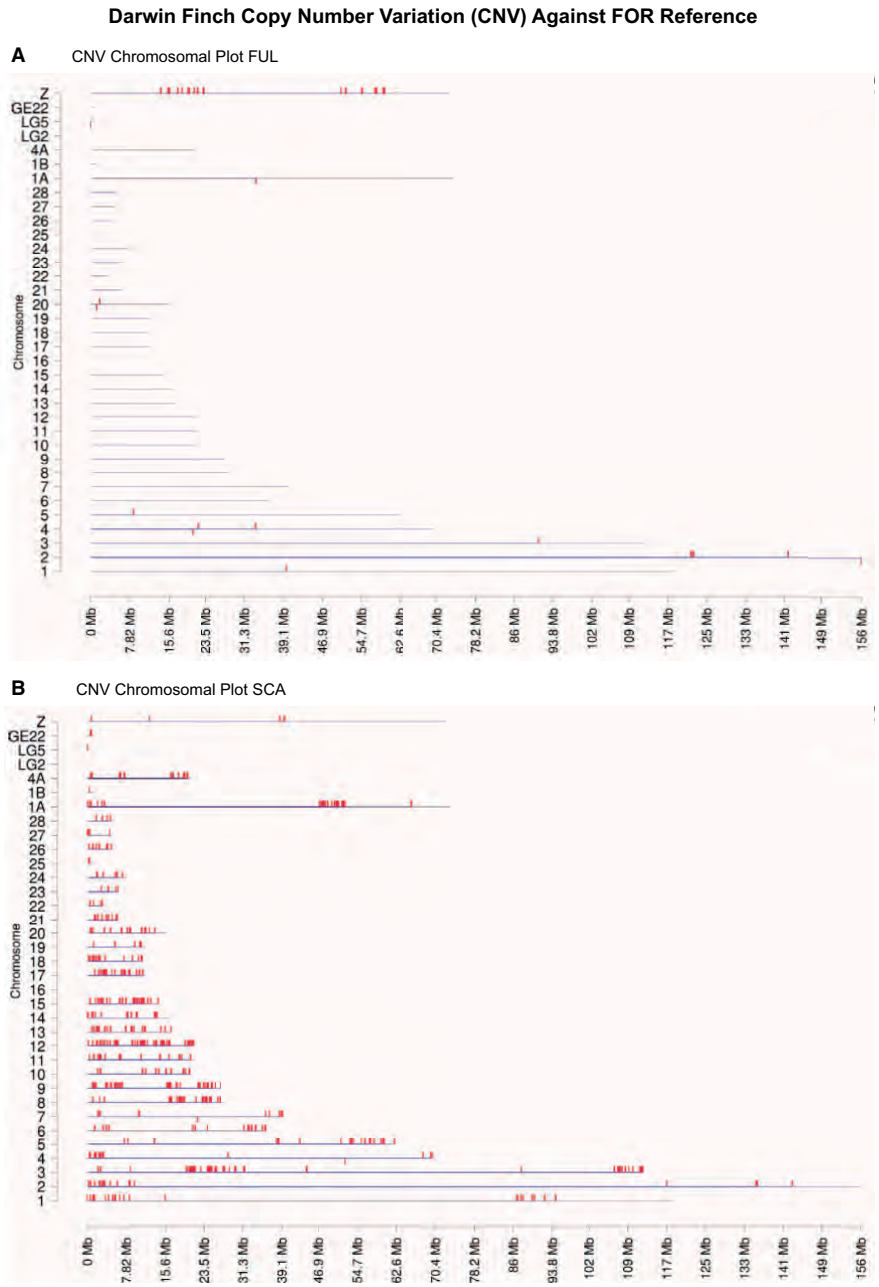


**Fig. 4.**—Chromosomal locations of the CNVs for each species. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of each CNV is marked with a red tick for FUL (*A*), SCA (*B*), PAR (*C*), and CRA (*D*).

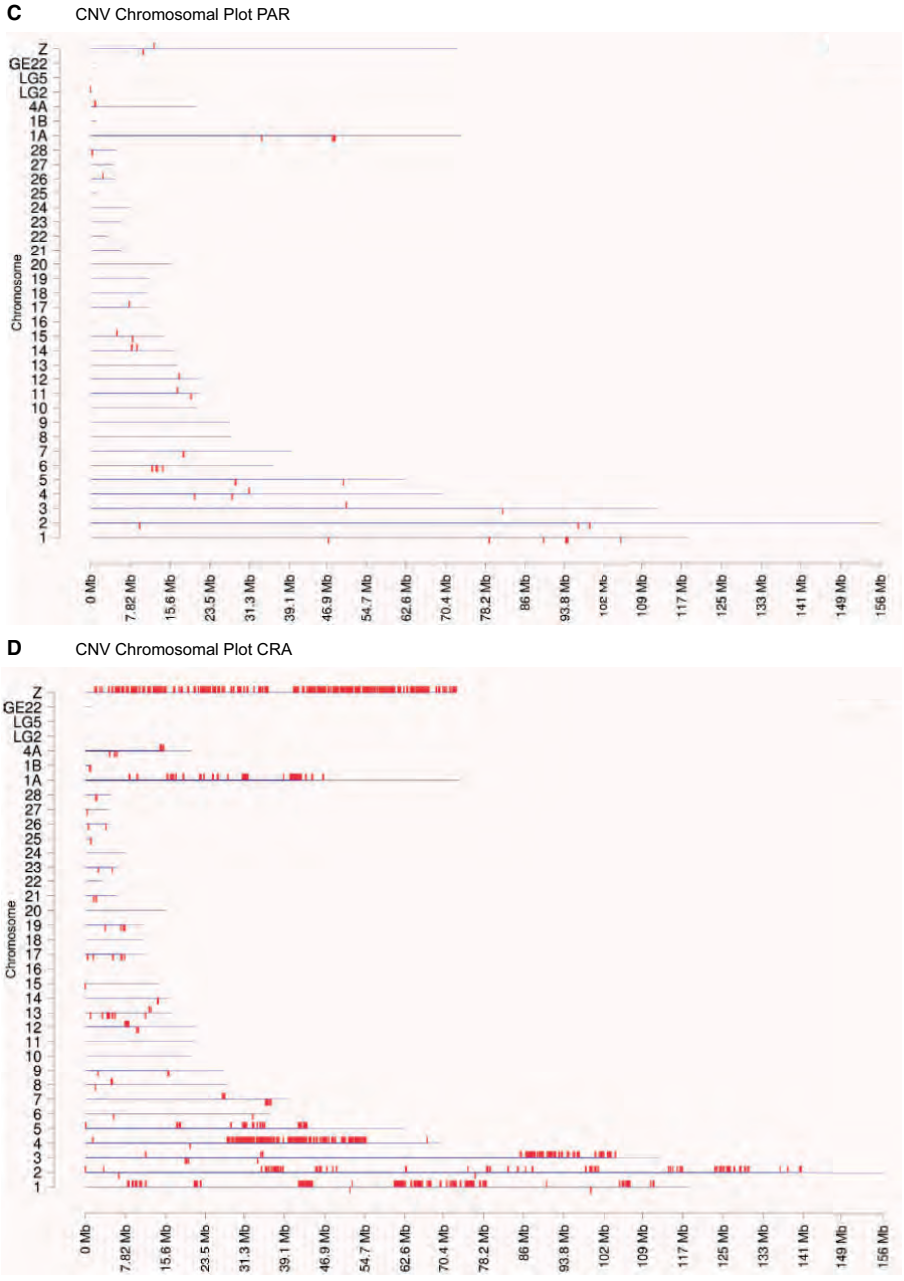


**Fig. 4.**—Continued.

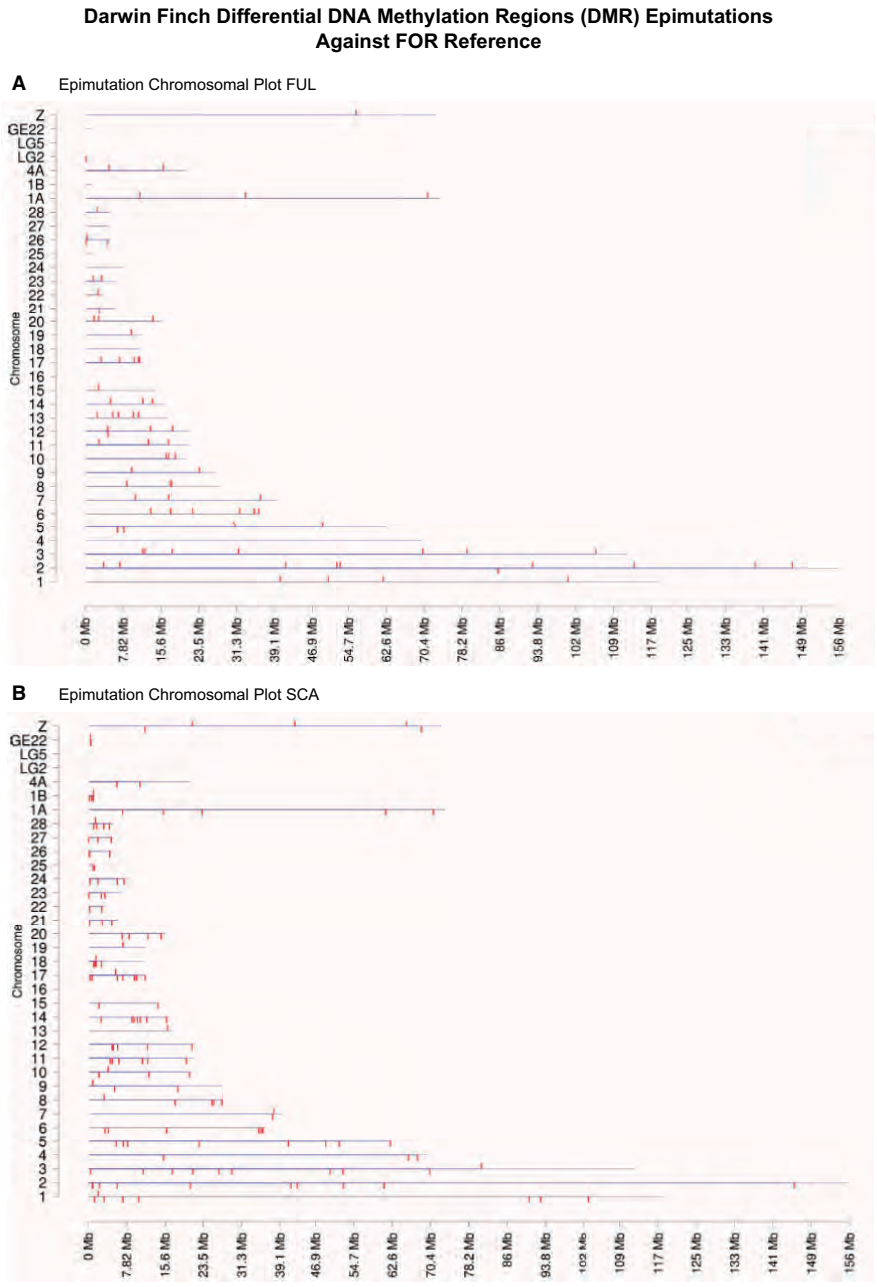


**Fig. 5.**—Chromosomal locations of the epimutations for each species. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of each DMR is marked with a red tick for FUL (*A*), SCA (*B*), PAR (*C*), and CRA (*D*).

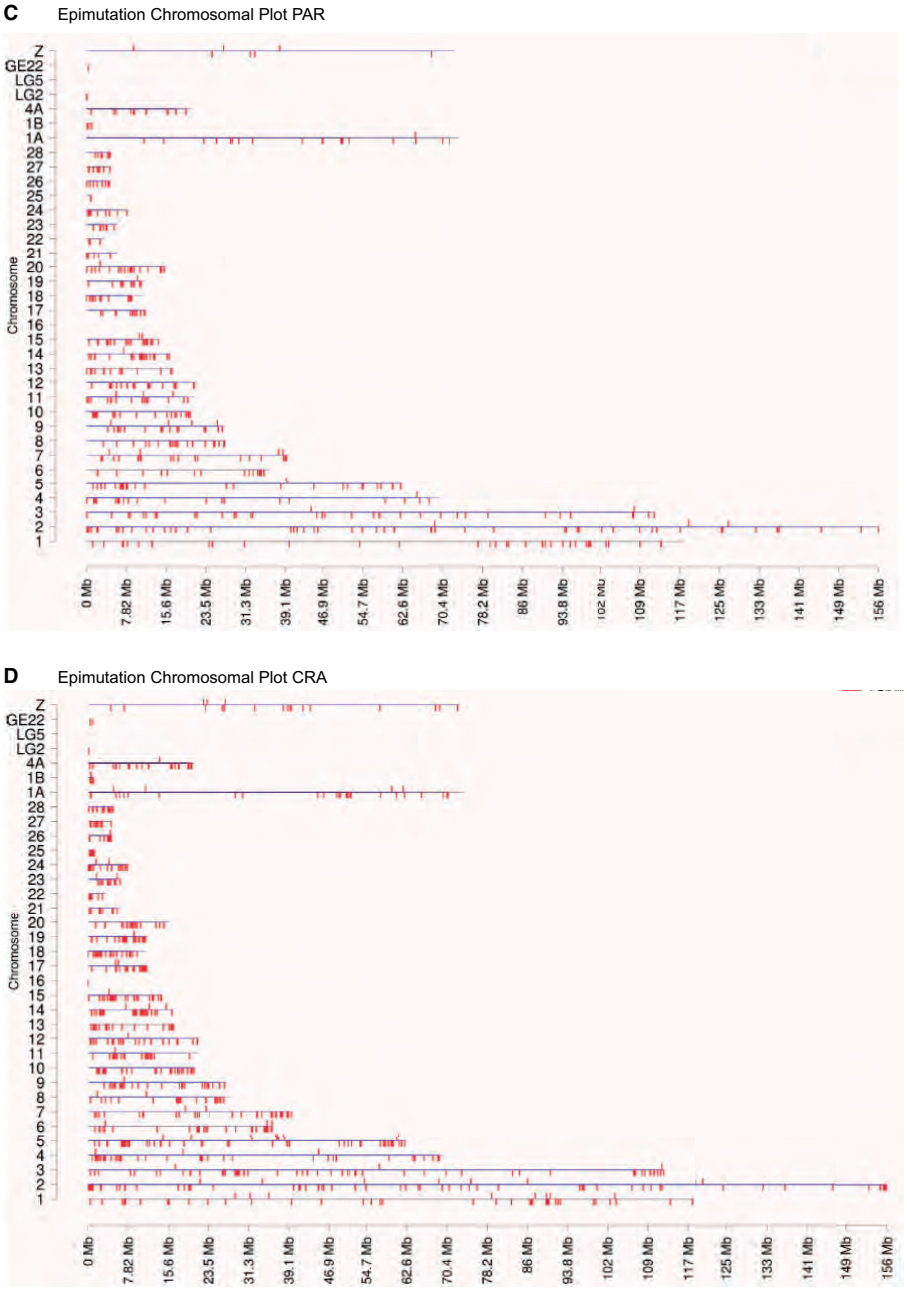


**Fig. 5.**—Continued.

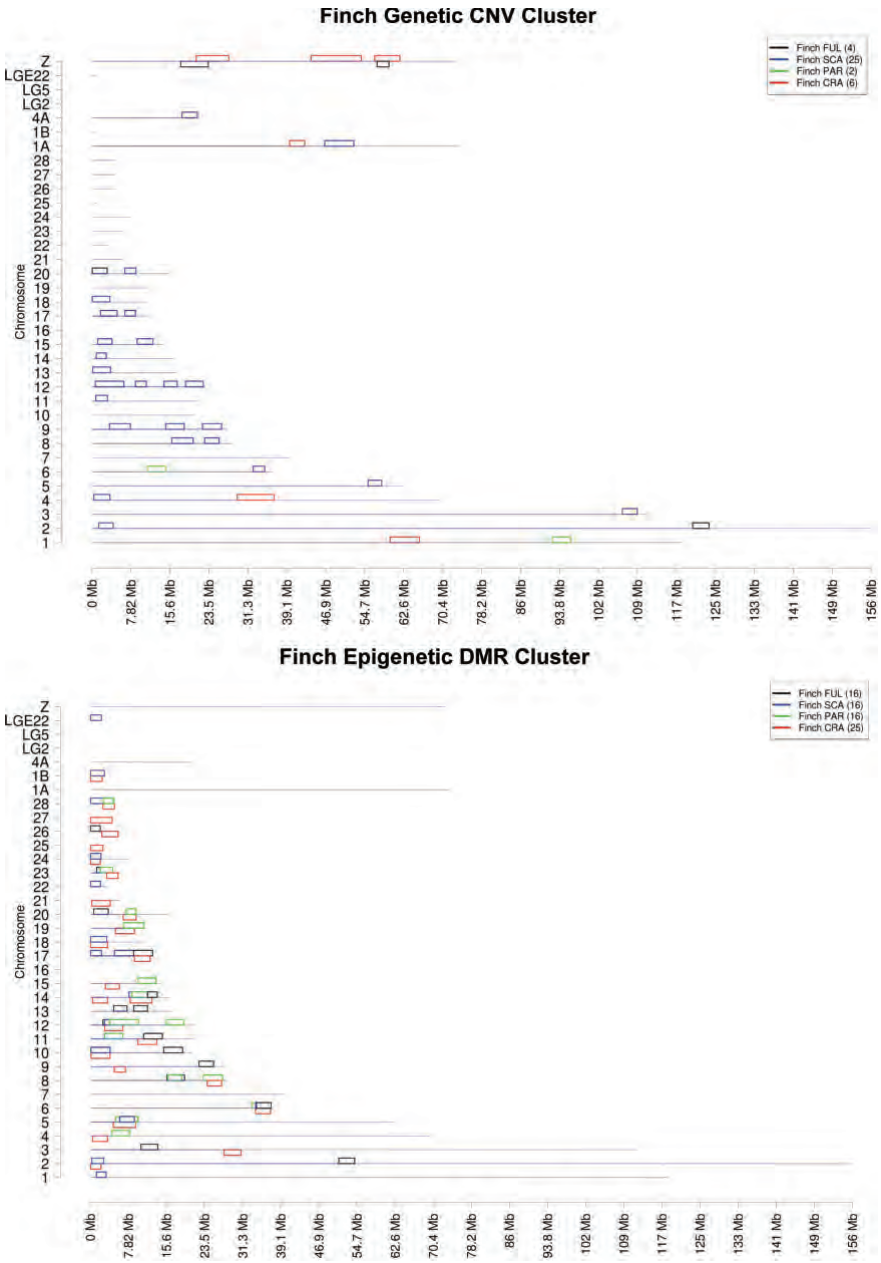


**Fig. 6.**—Chromosomal locations for clusters of CNV and DMR. The chromosome number and size are presented in reference to the zebra finch genome. The chromosomal location of statistically significant ($P < 10^{-5}$) overrepresented clusters of CNV (*A*) and DMR (*B*). The legend shows species and total number of clusters.

probes in the genome sequence having significant differential hybridization. These selection criteria reduce the number of false positives and provide a more reliable comparison (fig. 2). Therefore, the data presented used stringent criteria and represent the most reproducible epimutations and genetic CNV mutations among all three different experiments.

The increases or decreases in DNA methylation for the DMR are presented, along with the total number of epimutations in figure 2. The majority of epimutations for all species but FUL involves a decrease in DNA methylation (fig. 2*A*). The gains or losses in CNV are also presented, along with the total number of genetic alterations. The majority of genetic mutations for all species but PAR involves an increase in CNV number. Interestingly, the number of epimutations observed was generally higher, using the criteria selected, than the number of genetic alterations (fig. 2). However, the overall magnitude of epigenetic change was comparable to that of genetic change. Data for the five different species are shown in figure 1 for both epimutations (red) and genetic alterations (blue). The number of epimutations was significantly correlated with phylogenetic distance, whereas the number of genetic mutations was not (fig. 3).

The chromosomal locations of the CNV for the different finch species are shown in figure 4. CNVs were found on most chromosomes, with FUL having the least and CRA having the most. The chromosomal locations of the DMR epimutations for the different finch species are shown in figure 5. All chromosomes were found to have epimutations, with CRA having the highest number. These chromosomal plots suggested that some of the species might have clusters of CNV and/or DMR on some of the chromosomes (figs. 3 and 4). Therefore, a cluster analysis previously described (Skinner et al. 2012) was used to examine 50-kb regions throughout the genome to test for statistically significant ($P < 10^{-5}$) overrepresentation of CNV or DMR (fig. 6). Clusters, which have an average size of 3 Mb, are shown as species-specific boxes for CNV (fig. 6*A*) and for DMR (fig. 6*B*). Cluster characteristics and overlap are presented in supplementary table S3, Supplementary Material online. Clusters were obtained for all species, with a higher number of DMR clusters than CNV clusters. The highest number of CNV clusters was in SCA, with more than a 4-fold increase over CRA (fig. 6). Therefore, in addition to having more CNV than expected (assuming an increasing number with phylogenetic distance), SCA showed more CNV clusters than other species (fig. 2). Genome instability in these cluster regions may influence the increased numbers of CNV in SCA, which increases the presence of CNV clusters. In contrast, SCA did not show more DMR numbers or clusters than expected, assuming an increasing number with phylogenetic distance. Epimutation cluster overlap was more common among species (fig. 6 and table 1), suggesting that specific regions of the chromosomes were more susceptible to epigenetic alterations. Altered DNA methylation states have been experimentally shown to be stable for hundreds of generations (Cubas et al. 1999; Akimoto et al. 2007; Skinner et al. 2010).

**Table 1**
Cluster Overlap between Species

| **CNVs** | | | | |
|---|---|---|---|---|
| | **CNV** | | | |
| | **FUL** | **SCA** | **PAR** | **CRA** |
| FUL | 4 | 0 | 0 | 2 |
| SCA | 0 | 25 | 0 | 0 |
| PAR | 0 | 0 | 2 | 0 |
| CRA | 2 | 0 | 0 | 6 |
| **Epimutations** | | | | |
| | **DMR** | | | |
| | **FUL** | **SCA** | **PAR** | **CRA** |
| FUL | 16 | 5 | 6 | 7 |
| SCA | 5 | 16 | 8 | 11 |
| PAR | 6 | 8 | 16 | 11 |
| CRA | 7 | 11 | 11 | 25 |

Note.—The overlap of CNV or DMR clusters between species is presented for the CNVs and epimutations.

The potential overlaps in specific CNV or DMR sites among species were examined. The overlap in genetic mutations among the four species is shown in a Venn diagram in figure 2*C*, whereas the overlap in epimutations is shown in figure 2*B*. No overlap in specific CNV or DMR sites was observed among all species, and less than 10% overlap was generally observed between any two species. Interestingly, the CNV overlap between FUL and CRA was higher than for the other species (fig. 2*C*). Generally, genetic and epigenetic alterations were distinct between species, with the majority being species specific. The epimutations showed more overlap between species than the genetic CNV mutations (fig. 2*B* and table 1). In considering within species overlap between the CNV and epimutations, less than 3% had common genomic locations. Therefore, the epimutations do not appear to be linked to the genetic CNV mutations, but are distinct.

The final analysis examined the potential functional significance of the epimutations by examining DMR and genes known to be associated with avian evolution. Several gene families and cellular signaling pathways have previously been shown to be involved in bird evolution, including the bone morphogenic protein (BMP) family and pathway (Abzhanov et al. 2004; Badyaev et al. 2008), the toll receptor family and signaling pathway (Alcaide and Edwards 2011), and the melanins family and pathway (Mundy 2005). All the genes associated with these signaling pathways were localized on the finch genome and compared with the genomic locations of the epimutations and CNV. Epimutation-associated genes within the BMP pathway (fig. 7), toll pathway (fig. 8), and

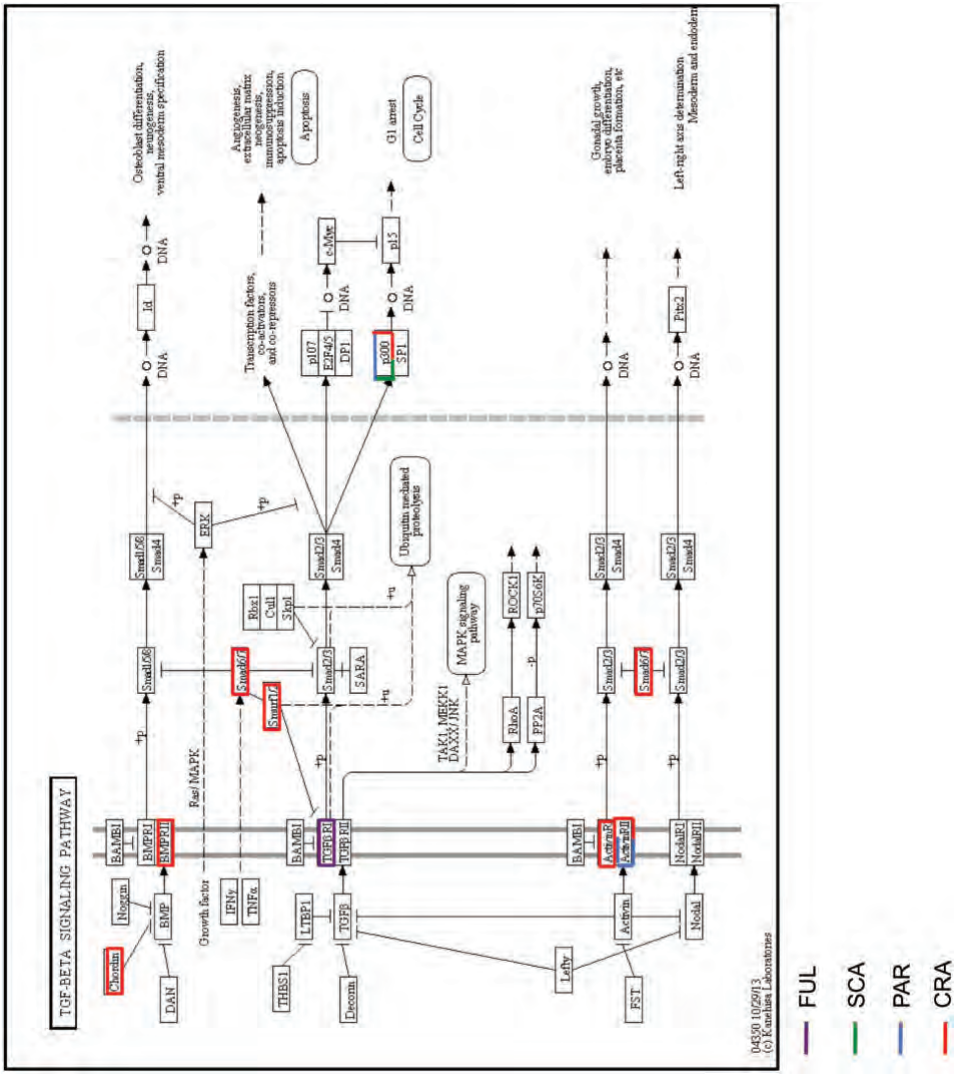


**Fig. 7.**—Epimutation-associated genes and correlated BMP pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

melanin's pathway (fig. 9) are shown. Epimutations were overrepresented in all of these pathways (Fisher's exact test: BMP/TGFbeta (transforming growth factor) pathway, $P < 1 \times 10^{-6}$; toll pathway, $P < 5.7 \times 10^{-4}$; melanogenesis pathway, $P < 2.5 \times 10^{-13}$). Interestingly, the BMP pathway involved in beak development and shape had a statistically significant overrepresentation of CRA-associated epimutations when examined independently ($P < 2.7 \times 10^{-5}$) (fig. 7). In addition, the toll receptor pathway involved in immune response had a statistically significant overrepresentation of PAR-associated epimutations when examined independently ($P < 7.7 \times 10^{-4}$) (fig. 8). The melanogenesis pathway involved in color had a mixture of epimutations from most of the species when examined independently ($P < 7 \times 10^{-5}$) (fig. 9).

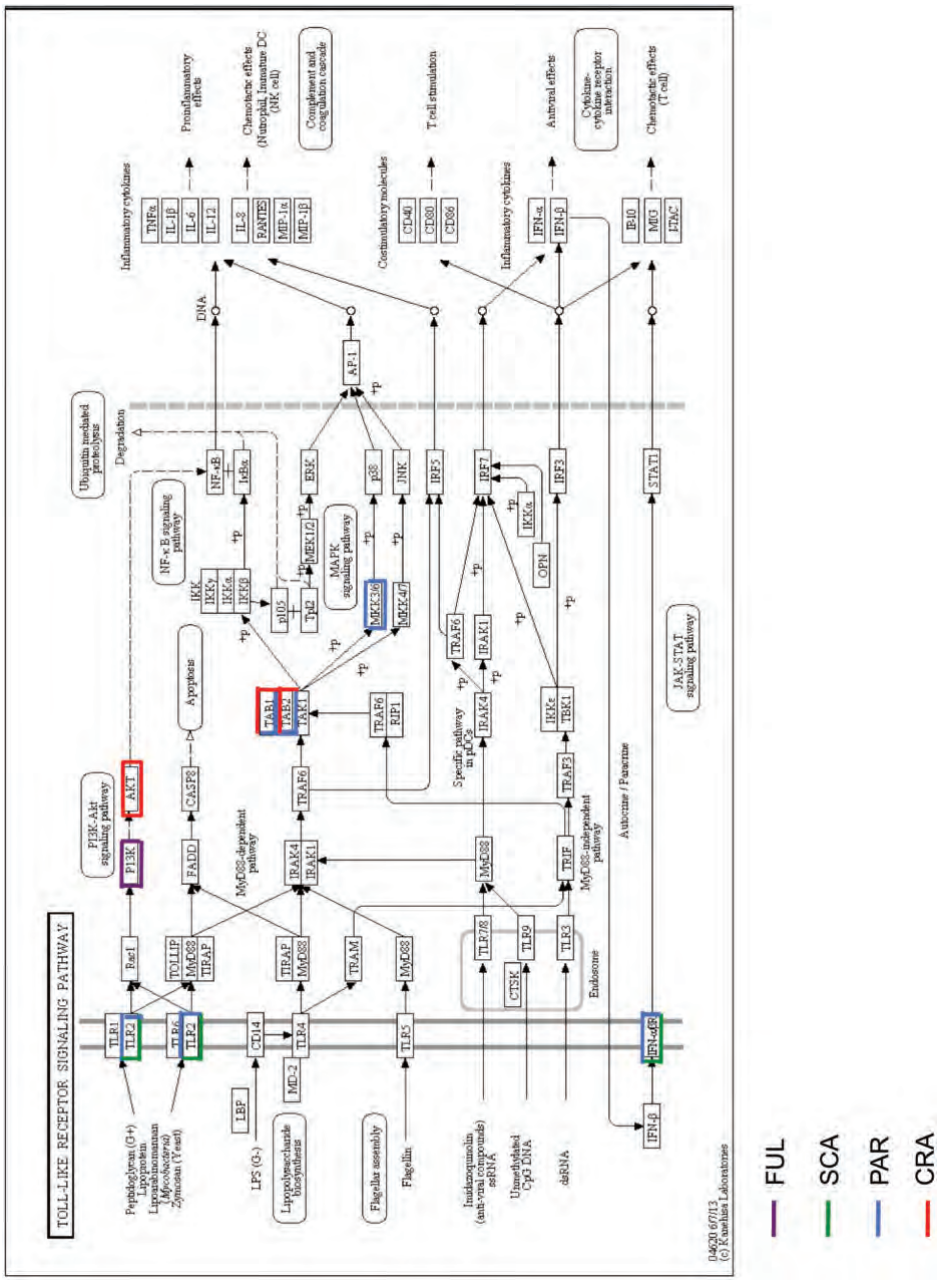


**Fig. 8.**—Epimutation-associated genes and correlated toll receptor pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

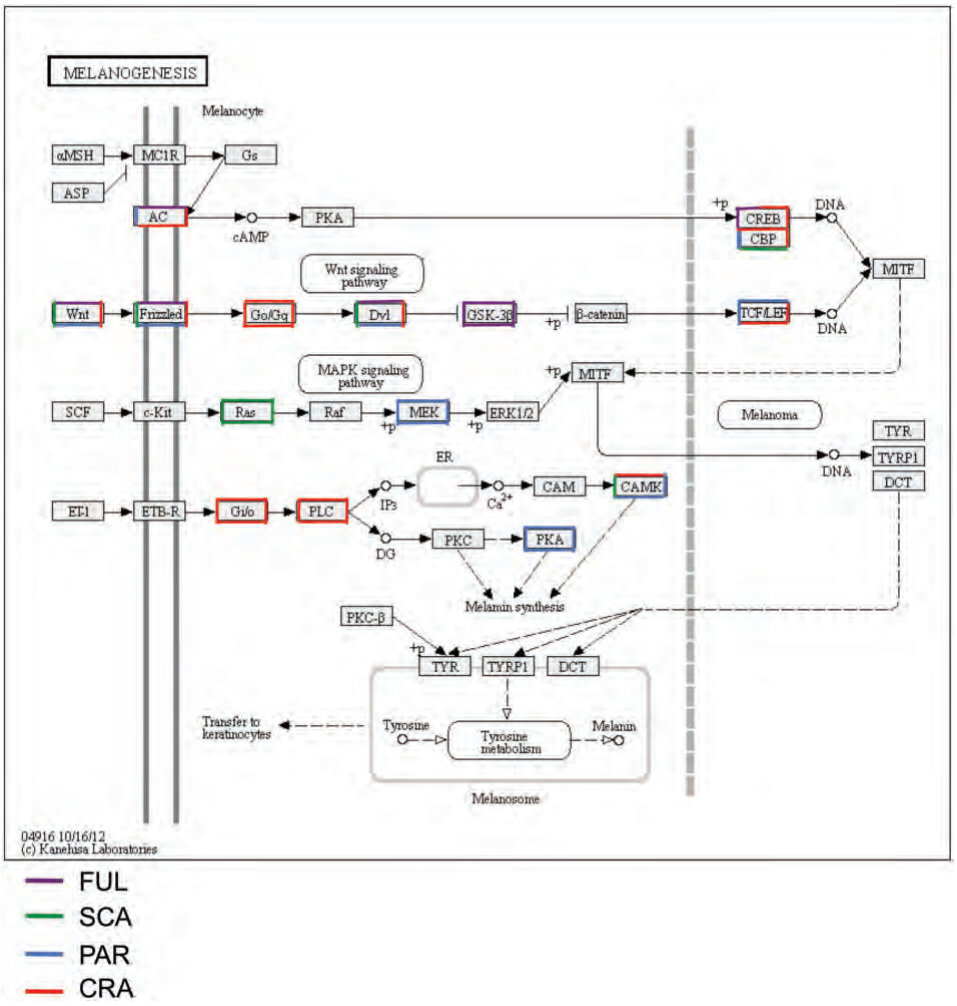


**Fig. 9.**—Epimutation-associated genes and correlated melanogenesis pathway. The genes having associated epimutations in the signaling pathway presented for the different species are identified as FUL (purple), SCA (green), PAR (blue), and CRA (red) colored boxed genes.

In addition to the pathway-specific genes, the total number of epimutations and CNV associated with genes are presented in table 2, with full lists in supplementary tables S4 and S5, Supplementary Material online. The epimutations and CNV for single probe and ≥3 probe identification are presented in table 2. Observations indicate that approximately half of the epimutations and CNV identified were associated with genes. Therefore, a high percentage of the epimutations and CNV identified were associated with genes and were statistically overrepresented in several gene pathways previously shown to be involved in particular aspects of avian evolution. Although this gene association analysis demonstrates that epimutations correlate with genes and important pathways, the functional or causal link to specific evolutionary processes remains to be investigated.

## Discussion

This study provides one of the first genome-wide comparisons of genetic and epigenetic mutations among related species of

**Table 2**
Epimutation and CNV Gene Associations

| CNVs | | | | |
|---|---|---|---|---|
| | Total CNV 1+ Probes | Total CNV 3+ Probes | CNV Association with 14K Genes 1+ Probes | CNV Association with 14K Genes 3+ Probes |
| FUL | 71 | 34 | 40 | 24 |
| SCA | 589 | 442 | 363 | 350 |
| PAR | 295 | 52 | 136 | 37 |
| CRA | 815 | 602 | 437 | 345 |
| **Epimutations** | | | | |
| | Total Epimutations 1+ Probes | Total Epimutations 3+ Probes | Epimutation Association with 14K Genes 1+ Probes | Epimutation Association with 14K Genes 3+ Probes |
| FUL | 514 | 84 | 295 | 48 |
| SCA | 890 | 161 | 558 | 115 |
| PAR | 1,629 | 606 | 996 | 407 |
| CRA | 2,767 | 1,062 | 1,611 | 639 |

NOTE.—The 14,000 zebra finch genes annotated having epimutation or CNV associations are presented for the total number of associations (overlaps) for both regions identified with single (1+ probes) and adjacent (3+ probes) data sets.

organisms. There were relatively more epimutations than genetic CNV mutations among the five species of Darwin's finches, which suggests that epimutations are a major component of genome variation during evolutionary change. There was also a statistically significant correlation between the number of epigenetic differences and phylogenetic distance between finches (figs. 1 and 3), indicating that the number of epigenetic changes continues to accumulate over long periods of evolutionary time (2–3 Myr). In contrast, there was no significant relationship between the number of genetic CNV changes and phylogenetic distance.

The zebra finch genome was used as a reference for this study because a complete Darwin's finch genome is not yet available. The zebra finch genome showed hybridization with all probes on the array for each of the Darwin's finch species, suggesting that the genomes appear to be extremely similar. Loss of heterozygosity (absence of genomic regions, resulting in lack of probe hybridization) was not identified in any of the analyses. This suggests a high level of conservation and identity between the species' genomes. In the event the Darwin's finch genome has additional DNA sequence that is not present in the zebra finch genome, we would not have detected this DNA. Therefore, our data may be an underestimate of the Darwin's finch genome. Another technical limitation of our study was that we only considered genetic CNV (amplifications and deletions of repeat elements), but not other genetic variants such as point mutations or translocations. Although CNV frequency is higher than other mutations (e.g., SNPs) and stable in the genome (Gazave et al. 2011), this study's focus on CNV should kept in mind. The epimutations examined are differential DMR that have previously been shown to be frequent and transgenerationally stable (Anway et al. 2005; Guerrero-Bosagna et al. 2010; Skinner et al. 2010). Although other epigenetic processes such as histone modification, altered chromatin structure, and noncoding RNA may also be important, DNA methylation is the most established heritable epigenetic mark. This aspect of the experimental design should be kept in mind.

Among the five species of finches there were fewer genetic mutations (CNV) than epigenetic mutations. However, the cactus finch SCA showed a surprisingly large number of genetic CNV mutations than expected when compared with the reference species (FOR). The SCA mutations also clustered to similar locations on the genome to a greater extent than in the other species (fig. 6*A*). The reason for the disproportionately large number of CNV in the SCA comparison is unclear.

In contrast to the genetic mutation (CNV) analysis, the number of epimutations increased monotonically with phylogenetic distance (figs. 1 and 3). Overlap of specific epigenetic sites among species was minimal, including those for SCA (fig. 2*B*). An interesting possibility is that the epigenome may alter genome stability and generate genetic variation within species. A similar phenomenon has been shown for cancer, in which epigenetic alterations may precede genetic changes and alter genomic stability (Feinberg 2004). A decrease in the DNA methylation of specific repeat elements has previously been shown to correlate with an increase in CNV (Macia et al. 2011; Tang et al. 2012). Therefore, environmentally induced abnormal epigenetic shifts may influence genetic

mutations, such that a combination of epigenetics and genetics promotes phenotypic variation. Our observations demonstrate a relationship between the number of epigenetic changes and phylogenetic distance.

A comparison of the positions of epimutations and known gene families was also carried out. These gene families included those involved in the BMP pathway, which is related to beak shape (Badyaev et al. 2008), the toll receptor pathway, which is involved in immunological function (Alcaide and Edwards 2011), and the melanogenesis pathway, which affects color (Mundy 2005). Genes in all three of these families and signaling pathways were found to have species-specific epimutations (figs. 7–9). Future studies should focus on the causal relationship between epigenetic alterations and phenotypic traits.

Genetic mutations are postulated to provide much of the variation upon which natural selection acts (Gazave et al. 2011; Stoltzfus 2012). However, genetic changes alone are limited in their ability to explain phenomena ranging from the molecular basis of disease etiology to aspects of evolution (Skinner et al. 2010; Day and Bonduriansky 2011; Longo et al. 2012; Klironomos et al. 2013). Therefore, genetic mutations may not be the only molecular factors to consider (Richards 2006, 2009). Indeed, epigenetic and genetic changes may jointly regulate genome activity and evolution, as recent evolutionary biology modeling suggests (Day and Bonduriansky 2011; Klironomos et al. 2013). This integration of genetics and epigenetics may improve our understanding of the molecular control of many aspects of biology, including evolution.

## Supplementary Material

Supplementary tables S1–S6 are available at *Genome Biology and Evolution* online (http://www.gbe. oxfordjournals.org/).

## Acknowledgments

The authors acknowledge the advice and critical reviews of Dr Jeb Owen (WSU), Dr Hubert Schwabl (WSU), Dr David Crews (U Texas Austin), Dr Kevin P. Johnson (U Illinois) and Dr Sarah Bush (Utah). They thank Ms Sean Leonard, Ms Shelby Weeks, Dr C. Le Bohec, Mr O. Tiselma and Mr R. Clayton for technical assistance and Ms Heather Johnson for assistance in preparation of the manuscript. The research was supported by the National Institute of Health grants to M.K.S. and National Science Foundation grants to D.H.C. M.K.S. conceived the study. M.K.S. and D.H.C. designed the study. C.G.B., M.M.H., E.E.N., J.A.H.K., S.A.K., and D.H.C. performed the experiments and acquired the data. All authors analyzed the data. M.K.S. and D.H.C. wrote the manuscript. All authors edited and approved the manuscript. The authors declare no competing financial interests.

## Literature Cited

Abzhanov A, Protas M, Grant BR, Grant PR, Tabin CJ. 2004. Bmp4 and morphological variation of beaks in Darwin's finches. Science 305(5689):1462–1465.

Akimoto K, et al. 2007. Epigenetic inheritance in rice plants. Ann Bot. 100(2):205–217.

Alcaide M, Edwards SV. 2011. Molecular evolution of the toll-like receptor multigene family in birds. Mol Biol Evol. 28(5):1703–1715.

Anway MD, Cupp AS, Uzumcu M, Skinner MK. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308(5727):1466–1469.

Badyaev AV, Young RL, Oh KP, Addison C. 2008. Evolution on a local scale: developmental, functional, and genetic bases of divergence in bill form and associated changes in song structure between adjacent habitats. Evolution 62(8):1951–1964.

Bonduriansky R. 2012. Rethinking heredity, again. Trends Ecol Evol. 27(6): 330–336.

Clayton DF, Balakrishnan CN, London SE. 2009. Integrating genomes, brain and behavior in the study of songbirds. Curr Biol. 19(18): R865–R873.

Crews D, et al. 2007. Transgenerational epigenetic imprints on mate preference. Proc Natl Acad Sci U S A. 104(14):5942–5946.

Cubas P, Vincent C, Coen E. 1999. An epigenetic mutation responsible for natural variation in floral symmetry. Nature 401(6749):157–161.

Day T, Bonduriansky R. 2011. A unified approach to the evolutionary consequences of genetic and nongenetic inheritance. Am Nat. 178(2):E18–E36.

Donohue K. 2011. Darwin's finches: readings in the evolution of a scientific paradigm. Chicago (IL): University of Chicago Press, p. 492.

Endler J. 1986. Natural selection in the wild. Princeton (NJ): Princeton University Press.

Feinberg AP. 2004. The epigenetics of cancer etiology. Semin Cancer Biol. 14(6):427–432.

Flatscher R, Frajman B, Schönswetter P, Paun O. 2012. Environmental heterogeneity and phenotypic divergence: can heritable epigenetic variation aid speciation? Genet Res Int. 2012:698421.

Gazave E, et al. 2011. Copy number variation analysis in the great apes reveals species-specific patterns of structural variation. Genome Res. 21(10):1626–1639.

Geoghegan JL, Spencer HG. 2012. Population-epigenetic models of selection. Theor Popul Biol. 81(3):232–242.

Geoghegan JL, Spencer HG. 2013a. Exploring epiallele stability in a population-epigenetic model. Theor Popul Biol. 83:136–144.

Geoghegan JL, Spencer HG. 2013b. The adaptive invasion of epialleles in a heterogeneous environment. Theor Popul Biol. 88:1–8.

Geoghegan JL, Spencer HG. 2013c. The evolutionary potential of paramutation: a population-epigenetic model. Theor Popul Biol. 88:9–19.

Grant P, Grant R. 2008. How and why species multiply: the radiation of Darwin's finches. Princeton (NJ): Princeton University Press.

Greenspan RJ. 2009. Selection, gene interaction, and flexible gene networks. Cold Spring Harb Symp Quant Biol. 74:131–138.

Guerrero-Bosagna C, Sabat P, Valladares L. 2005. Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos? Evol Dev. 7(4):341–350.

Guerrero-Bosagna C, Settles M, Lucker B, Skinner MK. 2010. Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. PLoS One 5(9):e13100.

Holeski LM, Jander G, Agrawal AA. 2012. Transgenerational defense induction and epigenetic inheritance in plants. Trends Ecol Evol. 27: 618–626.

Huber SK, et al. 2010. Ecoimmunity in Darwin's finches: invasive parasites trigger acquired immunity in the medium ground finch (*Geospiza fortis*). PLoS One 5(1):e8605.

Huttley GA. 2004. Modeling the impact of DNA methylation on the evolution of BRCA1 in mammals. Mol Biol Evol. 21(9):1760–1768.
Jirtle RL, Skinner MK. 2007. Environmental epigenomics and disease susceptibility. Nat Rev Genet. 8(4):253–262.
Klironomos FD, Berg J, Collins S. 2013. How epigenetic mutations can affect genetic evolution: model and mechanism. Bioessays 35(6): 571–578.
Koop JA, Huber SK, Laverty SM, Clayton DH. 2011. Experimental demonstration of the fitness consequences of an introduced parasite of Darwin's finches. PLoS One 6(5):e19706.
Kuzawa CW, Thayer ZM. 2011. Timescales of human adaptation: the role of epigenetic processes. Epigenomics 3(2):221–234.
Lack D. 1947. Darwin's finches. Cambridge University Press.
Lamarck JB. 1802. Recherches sur l'organisation des corps vivans. Paris: Chez L'auteur, Maillard.
Liebl AL, Schrey AW, Richards CL, Martin LB. 2013. Patterns of DNA methylation throughout a range expansion of an introduced songbird. Integr Comp Biol. 53(2):351–358.
Longo G, Miquel PA, Sonnenschein C, Soto AM. 2012. Is information a proper observable for biological organization? Prog Biophys Mol Biol. 109(3):108–114.
Lupski JR. 2007. An evolution revolution provides further revelation. Bioessays 29(12):1182–1184.
Macia A, et al. 2011. Epigenetic control of retrotransposon expression in human embryonic stem cells. Mol Cell Biol. 31(2):300–316.
Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK. 2012. Transgenerational actions of environmental compounds on reproductive disease and epigenetic biomarkers of ancestral exposures. PLoS One 7(2):e31901.
Mundy NI. 2005. A window on the genetics of evolution: MC1R and plumage colouration in birds. Proc Biol Sci. 272(1573):1633–1640.
Nozawa M, Kawahara Y, Nei M. 2007. Genomic drift and copy number variation of sensory receptor genes in humans. Proc Natl Acad Sci U S A. 104(51):20421–20426.
Olshen AB, Venkatraman ES, Lucito R, Wigler M. 2004. Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 5(4):557–572.
Petren K, Grand BR, Grant PR. 1999. A phylogeny of Darwin's finches based on microsatellite DNA lenght variation. Proc R Soc Lond B. 266(1417):321–329.
Picard F, Robin S, Lavielle M, Vaisse C, Daudin J-J. 2005. A statistical approach for array CGH data analysis. BMC Bioinformatics 6:27.
Pinkel D, Albertson DG. 2005. Comparative genomic hybridization. Annu Rev Genomics Hum Genet. 6:331–354.
Poptsova M, Banerjee S, Gokcumen O, Rubin MA, Demichelis F. 2013. Impact of constitutional copy number variants on biological pathway evolution. BMC Evol Biol. 13:19.
R Development Core Team. 2010. R: a language for statistical computing. Vienna (Austria): R Foundation for Statistical Computing. Available from: http://www.R-project.org.
Rands CM, et al. 2013. Insights into the evolution of Darwin's finches from comparative analysis of the *Geospiza magnirostris* genome sequence. BMC Genomics 14:95.
Rebollo R, Horard B, Hubert B, Vieira C. 2010. Jumping genes and epigenetics: towards new species. Gene 454(1–2):1–7.
Richards CL, Bossdorf O, Pigliucci M. 2010. What role does heritable epigenetic variation play in phenotypic evolution? BioScience 60: 232–237.
Richards EJ. 2006. Inherited epigenetic variation—revisiting soft inheritance. Nat Rev Genet. 7(5):395–401.
Richards EJ. 2009. Quantitative epigenetics: DNA sequence variation need not apply. Genes Dev. 23(14):1601–1605.
Skinner MK. 2011. Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. Epigenetics 6(7): 838–842.
Skinner MK, Anway MD, Savenkova MI, Gore AC, Crews D. 2008. Transgenerational epigenetic programming of the brain transcriptome and anxiety behavior. PLoS One 3(11):e3745.
Skinner MK, Manikkam M, Guerrero-Bosagna C. 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. Trends Endocrinol Metab. 21(4):214–222.
Skinner MK, Mohan M, Haque MM, Zhang B, Savenkova MI. 2012. Epigenetic transgenerational inheritance of somatic transcriptomes and epigenetic control regions. Genome Biol. 13(10):R91.
Skinner MK, Savenkova MI, Zhang B, Gore AC, Crews D. 2014. Gene bionetworks involved in epigenetic transgenerational inheritance of altered mate preference: environmental epigenetics and evolutionary biology. BMC Genomics 15:377.
Slatkin M. 2009. Epigenetic inheritance and the missing heritability problem. Genetics 182(3):845–850.
Stoltzfus A. 2012. Constructive neutral evolution: exploring evolutionary theory's curious disconnect. Biol Direct. 7:35.
Sudmant PH, et al. 2013. Evolution and diversity of copy number variation in the great ape lineage. Genome Res. 23:1373–1382.
Tang MH, et al. 2012. Major chromosomal breakpoint intervals in breast cancer co-localize with differentially methylated regions. Front Oncol. 2:197.
Tateno H, Kimura Y, Yanagimachi R. 2000. Sonication per se is not as deleterious to sperm chromosomes as previously inferred. Biol Reprod. 63(1):341–346.
Tibshirani R, Wang P. 2008. Spatial smoothing and hot spot detection for CGH data using the fused lasso. Biostatistics 9(1):18–29.
Ward WS, Kimura Y, Yanagimachi R. 1999. An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. Biol Reprod. 60(3):702–706.
Whitlock MC, Schluter D. 2009. The analysis of biological data. Greenwood Village (CO): Roberts and Company Publishers.
WUSTL. 2008. Jul. 2008 assembly of the zebra finch genome (taeGut1, WUSTL v3.2.4), as well as repeat annotations and GenBank sequences, Database Provider, NCBI.
Ying H, Huttley G. 2011. Exploiting CpG hypermutability to identify phenotypically significant variation within human protein-coding genes. Genome Biol Evol. 3:938–949.

**Associate editor:** Bill Martin

# APPENDIX C

# DOES AVIAN MALARIA REDUCE FLEDGING SUCCESS: AN EXPERIMENTAL TEST OF THE SELECTION HYPOTHESIS

Printed with permission from:
Knutie, S.A., J.L. Waite, D.H. Clayton. 2013. Does avian malaria reduce fledging success: An experimental test of the selection hypothesis. Evolutionary Ecology 27: 185-191, doi:10.1007/s10682-012-9578-y.

Evol Ecol
DOI 10.1007/s10682-012-9578-y

ORIGINAL PAPER

# Does avian malaria reduce fledging success: an experimental test of the selection hypothesis

**Sarah A. Knutie · Jessica L. Waite · Dale H. Clayton**

Received: 13 February 2012 / Accepted: 10 May 2012


**Abstract** Like many parasites, avian haematozoa are often found at lower infection intensities in older birds than young birds. One explanation, known as the "selection" hypothesis, is that infected young birds die before reaching adulthood, thus removing the highest infection intensities from the host population. We tested this hypothesis in the field by experimentally infecting nestling rock pigeons *(Columba livia)* with the malaria parasite *Haemoproteus columbae*. We compared the condition and fledging success of infected nestlings to that of uninfected controls. There was no significant difference in the body mass, fledging success, age at fledging, or post-fledging survival of experimental versus control birds. These results were unexpected, given that long-term studies of older pigeons have demonstrated chronic effects of *H. columbae*. We conclude that *H. columbae* has little impact on nestling pigeons, even when they are directly infected with the parasite. Our study provides no support for the selection hypothesis that older birds have lower parasite loads because parasites are removed from the population by infected nestlings dying. To our knowledge, this is the first study to test the impact of avian malaria using experimental inoculations under natural conditions.

**Keywords** *Columba livia* · Pigeon · Fitness · Hippoboscid fly · Host-parasite interaction

## Introduction

Parasites influence fundamental aspects of the evolutionary ecology of their hosts, such as population dynamics (Anderson and May 1978; Anderson 1979) and life history evolution (Hochberg et al. 1992). The impact of parasites on host fitness depends partly on the age at which hosts become infected. A common pattern in host-parasite interactions is that

Sarah A. Knutie and Jessica L. Waite contributed equally to this work.

S. A. Knutie · J. L. Waite (✉) · D. H. Clayton
Biology Department, University of Utah, Salt Lake City, UT 84112, USA
e-mail: jessi.waite@gmail.com

Published online: 24 May 2012

younger individuals have higher parasite loads than adults (Gregory et al. 1992; Hudson and Dobson 1997). Sol et al. (2003) considered three hypotheses to explain this pattern. The "selection" hypothesis suggests that highly parasitized juvenile hosts die before they reach adulthood, removing large numbers of parasites from the population. The "immunity" hypothesis suggests that the developing immune system of juveniles is not yet capable of killing parasites, while adults are much more effective at reducing parasite intensity. The "vector exposure" hypothesis suggests that adult behavior reduces their exposure to infected vectors, and thus parasites, compared to juveniles.

Sol et al. (2003) evaluated these hypotheses using data from a study of feral rock pigeons *(Columba livia)* infected with malaria parasites *(Haemoproteus columbae)* vectored by pigeon louse flies *(*Hippoboscoidea: *Pseudolynchia canariensis).* The authors rejected the vector exposure hypothesis because they found that adult pigeons (>6 months old) are not, in fact, exposed to fewer vectors than juvenile pigeons (Sol et al. 2000). Although the authors reported higher rates of juvenile mortality (61 %) compared to adult mortality (33 %), consistent with the selection hypothesis, selection in their study was not strong enough to explain the lower number of parasites observed in adult birds. The youngest birds in Sol et al.'s study had already fledged from the nest; however, the greatest impact of *H. columbae* on pigeons may occur while birds are still in the nest. We conducted a study to test the impact of *H. columbae* on the condition and fledging success of younger, nestling rock pigeons. We used an experimental approach in which we compared nestlings injected with *H. columbae* to control birds not injected with the parasite.

At least 200 species of *Haemoproteus* are known to infect birds worldwide (Martinsen et al. 2008). Perez-Tris et al. (2005) classified *Haemoproteus* as an avian malaria parasite because members of the genus were nested phylogenetically within the genus *Plasmodium*. *H. columbae* is a parasite of pigeons and doves that uses blood-feeding pigeon flies as vectors (Valkiūnas 2005). The parasite enters a feeding fly and reproduces in its midgut, where *H. columbae* oocysts attach to the gut wall. Once mature, the oocysts burst and release infective sporozoites that migrate from the fly's gut into its salivary glands. The fly then injects these sporozoites into a pigeon when it feeds. *H. columbae* reproduces asexually in the lungs of the pigeon, then invades and matures in the red blood cells (Ahmed and Mohammed 1978).

*Haemoproteus* species can have several negative effects on host fitness. These effects include reductions in host body condition (Merino et al. 2000), lower reproductive success (Marzal et al. 2004; Tomas et al. 2007), and even death (Atkinson and Forrester 1988; Sol et al. 2003). Studies of the impact of malaria on juvenile birds have consisted of observational studies in the field (Sol et al. 2003), and experimental studies using captive birds (Yorinks and Atkinson 2000; Garvin et al. 2003). The goal of our study was to use an experimental approach under field conditions. We infected nestling birds with malaria parasites to test the impact on body mass, fledging success, age at fledging, and post-fledging survival of experimental versus control birds. Studies with captive birds suggest that the most pathogenic phase of the *Haemoproteus* life cycle occurs when parasites enter red blood cells to mature (Atkinson and Forrester 1988; Atkinson and van Riper 1991). In the case of *H. columbae* this takes place about 24–37 days after infection (Ahmed and Mohammed 1978). Since pigeons fledge at about 32 days of age, it is not possible to be sure that fledglings are infected with malaria parasites, short of experimentally infecting them. Experimental manipulation is the most powerful approach for testing the impact of parasites on hosts in any case (McCallum and Dobson 1995). To our knowledge, this is the first study to test the impact of avian malaria parasites using experimental inoculation under natural conditions.

## Materials and methods

We experimentally manipulated *H. columbae* in nestling rock pigeons. The study took place August–November 2009 under a highway overpass in Draper, Utah, USA (40°31′36″N, 111°53′28″W). We visited the field site every 2–3 days throughout the study period. Nestlings were weighed at each visit to the nearest 1.0 g with a pesola scale. Our experiment was restricted to nests with two nestlings, the normal number for rock pigeons. Nests were sequentially assigned to one of three treatment groups: experimental (n = 12 nests), control (n = 13), or background (n = 12). When nestlings were 4–7 days old (50–150 g), those at experimental nests were injected with a suspension of *P. canariensis* flies infected with *H. columbae* (Ahmed and Mohammed 1978). We created the infected fly suspension by feeding flies (bred from wild stock) on heavily infected captive birds. Following 10–12 days on a bird, flies were placed in vials and taken to the field site, where batches of ten live flies were macerated in 1,000 μL of phosphate buffered saline for 3 min. Experimental nestlings were injected intraperitoneally with 500 μL of the infected fly suspension using a 0.5 cc syringe. Control birds were injected with 500 μL of another suspension made using uninfected flies. Background birds were handled but not injected.

Prior to the field experiment, we conducted a test of the inoculation method using 27 wild trapped, captive rock pigeons. After blocking by capture date and site, 13 randomly chosen birds were injected with a suspension of infected flies, as described above. Fourteen control birds were injected with a suspension of uninfected flies. At 25, 35, and 42 days post injection, blood samples were taken from all birds and smears were prepared for examination. Each smear was carefully examined under oil immersion at 1,000 × for 10 min; if parasites were detected, then the number of parasites was quantified in 25 microscope fields per bird. All 13 experimental birds were infected with *H. columbae*, while none of the 14 control birds was infected.

When nestlings were approximately 10 days old they were fitted with a numbered aluminum band and three plastic color bands. To score fledging success we observed and identified birds after they left the nest on the basis of their color band combinations. We conducted a thorough census of all birds at the bridge during each visit to the field site. We also searched for banded birds at other bridges within 8 km of the study site in order to determine whether newly fledged birds were dispersing from the natal site.

We continued to monitor birds at the bridge for 50 days post injection (ca. 25 days post fledging) because peak parasitemia can be delayed for this long after injection (extrapolated from Ahmed and Mohammed 1978). To confirm experimental infections, we examined the blood of birds after they fledged. We used walk-in traps to capture pigeons from 30–50 days post injection. Blood samples were taken and birds immediately released. Blood smears were prepared and examined back in the lab.

Data were analyzed using Prism® v.5.0b (GraphPad Software, Inc.). Power analyses were conducted in G*Power 3 with an error probability set at 0.05 (Buchner et al. 1997). Where necessary, data were log transformed for normalization. To avoid pseudoreplication (Hurlbert 1984) we averaged values for nestlings within each nest. We used one-way ANOVAs to compare parasite abundance and host age and mass at fledging among treatments. A repeated-measures ANOVA was used to compare the number of birds per nest at hatching, fledging, and 1, 2, and 3 weeks post-fledging.

## Results

Three times as many experimental birds were infected as control or background birds (Fig. 1a); the three groups also differed in parasite abundance (Fig. 1b; ANOVA $F_{2,17} = 4.25$, $P < 0.05$). Dunnett's post hoc comparisons confirmed that experimental birds had significantly more parasites than controls ($P < 0.05$), while control and background birds did not differ significantly ($P > 0.05$).

There was no significant difference in the age of birds at fledging, nor body mass prior to fledging (see Table 1). There was no significant difference in the proportion of nests that fledged at least one offspring ($\chi^2 = 0.005$, $P = 0.99$). There was also no significant effect of treatment on the mean number of birds fledged per nest, nor the number of birds observed after fledging (Fig. 2; repeated measures ANOVA, treatment $F_{2,34} = 0.64$, $P = 0.53$). There was a significant effect of time (Fig. 2; time, $F_{4,136} = 43.32$, $P < 0.0001$), but no significant interaction between time and treatment (time*treatment, $F_{8,136} = 0.49$, $P = 0.86$).

We reanalyzed the data after excluding naturally infected control and background birds, as well as experimental birds for which we could not confirm infection. We still found no significant difference in age at fledging ($F_{2,31} = 0.53$, $P = 0.60$) mass at fledging ($F_{2,31} = 1.01$, $P = 0.38$), or the proportion of nests that fledged at least one offspring ($\chi^2 = 0.01$, $P = 0.99$).

Our experiment had considerable power (1.0) to detect the level of juvenile mortality (61 %) reported by Sol et al. (2003), we had power of 0.8 to detect mortality of at least 30 % (effect size of f = 0.55).

## Discussion

Our goal was to experimentally test the "selection" hypothesis. This hypothesis, reviewed by Gregory et al. (1992), states that lower parasite loads of adults, compared to juveniles, are the result of heavily infected juveniles dying before adulthood, removing parasites from the population. Previous tests of this hypothesis involving avian malaria have focused on juvenile (fledged) birds and relied on observational data (Sol et al. 2003;

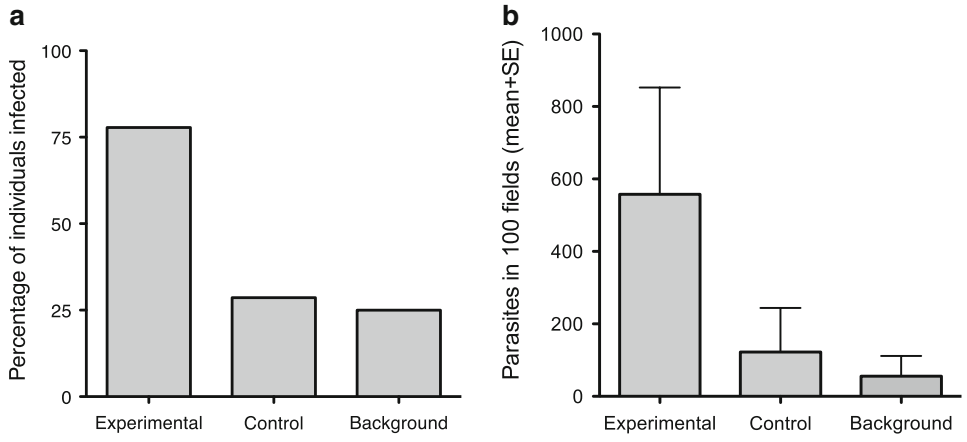


**Fig. 1** Prevalence **(a)** and mean abundance (+SE) **(b)** of malaria parasites 30–50 days after treatment

**Table 1** Age of birds at fledging and body mass prior to fledging. Values are grand means (±SE) of the mean value per nest

| | Experimental | Control | Background | Test statistic | *P* |
|---|---|---|---|---|---|
| Age in days | 32.3 ± 0.5 | 31.8 ± 0.6 | 32.3 ± 0.6 | $F = 0.25$ | 0.78 |
| (Number of nests) | (11) | (12) | (11) | | |
| Mass in grams | 298 ± 14.9 | 311.3 ± 12.5 | 313.1 ± 11.4 | $F = 0.35$ | 0.71 |
| (Number of nests) | (11) | (12) | (11) | | |

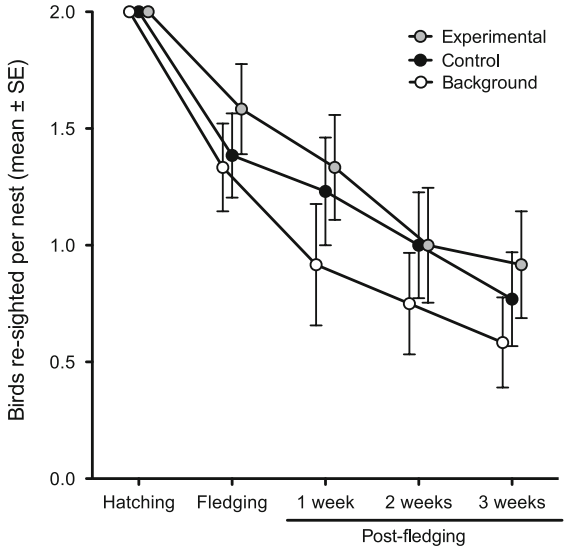


**Fig. 2** Mean (±SE) offspring observed per nest. The mean (±SE) number of offspring fledged per nest did not differ significantly among treatments. Points are slightly offset for clarity

van Oers et al. 2010). These studies provided some support for the selection hypothesis, but the intensity of selection measured could not fully explain differences in juvenile and adult parasite loads. It was conceivable, therefore, that the greatest impact of *H. columbae* on pigeons takes place while they are still in the nest.

Our results provided no support for the selection hypothesis because there was no impact of malaria on any of the components of host fitness we measured. Specifically, there was no significant difference in the body mass, fledging success, age at fledging, or post-fledging survival of experimental versus control birds. We are confident that our measures of post-fledging survival were accurate because none of the birds from our study were observed at other bridges (see methods). Young pigeons do not normally disperse until 3 months of age, in any case (Johnston and Janiga 1995).

The results of our study were unexpected, given that Sol et al.'s longer-term study demonstrated that *H. columbae* has a significant negative impact on pigeon fitness. The fact that malaria had no detectable impact on fledging success in our study was not due to unusually low rates of fledging in both experimental and control birds. Fledging success was 73 % (Fig. 2), similar to that in other studies of feral pigeons [reviewed by Johnston and Janiga (1995), Table 18.4 (values adjusted for hatching rates)]. Similarly, the fact that malaria had no detectable impact on fledging was not due to methodological problems with the creation of experimental infections. The malaria parasite levels in our study were comparable to those observed in other studies of naturally infected pigeons (Kartman 1949;

Klei and DeGuisti 1975; Paperna and Smallridge 2002). However, *H. columbae* may affect hosts only at levels higher than what we observed (Earle et al. 1993; Paperna and Smallridge 2002). For example, the *H. columbae* levels in Sol et al.'s (2003) study were among the highest ever recorded for feral rock pigeons.

Another factor that could conceivably contribute to why the birds in our study did not appear to be affected by *H. columbae*, compared to the reduction in survival shown for older birds by Sol et al. (2003), is that nestling pigeons could have higher tolerance to parasites than older birds. Nestlings are fed a rich diet of crop milk by both parents. The milk, which consists of the sloughed lining of the parents' crop, is very high in fat and protein (Johnston and Janiga 1995). It would be interesting to test the impact of *H. columbae* on nestlings fed a less nutritious diet.

A few control and background birds were naturally infected with *H. columbae*. However, infection levels were still significantly higher in the experimental group than the control or background groups. Even after excluding the naturally infected birds, we did not find that malaria parasites affected fledging age or mass, or fledging success.

Since *H. columbae* had no apparent effect on nestling rock pigeons, our study does not provide support for the "selection hypothesis". Sol et al. (2003) reported results that were consistent with selection hypothesis; however, selection in their study was not strong enough to explain the differences in parasitemia they observed between juvenile and adult pigeons. Because Sol et al. (2000, 2003) reported data ruling out the "vector exposure" hypothesis, they suggested a combination of the selection and immunity hypotheses may explain the fact that juvenile birds have higher parasitemia than adult birds. Our data provide no reason to disagree with this assessment.

To our knowledge, this is the first study to test the impact of avian malaria parasites using experimental inoculation under natural conditions. This approach has several advantages. First, like many malaria parasites, *H. columbae* takes several weeks to appear in the peripheral blood after the host is infected. This fact makes early infections difficult to detect without more invasive methods, such as collection of organ tissues (Valkiūnas 2005; Cosgrove et al. 2006). Experimental infections get around this problem. Second, inoculating hosts with parasites has the strong advantage of controlling for factors that could lead to spurious negative correlations between parasite load and host fitness (Hawlena et al. 2006; Blanchet et al. 2009). The greatest limitation of our study is that the modest sample sizes limit our ability to detect relatively small effect of malaria parasites on birds. For example, to detect a 10 % reduction in juvenile survival with a power of 0.8 would require a sample of 93 nests per treatment for a total of 279 nests. A study of this magnitude may be feasible in the future using feral Rock Pigeons and *H. columbae.*

**Acknowledgments** We thank Blair Racker, Dallas Brewer, Joseph Flower, Autumn Henry, Corbin Smith, Scott Villa, and Jennifer Koop for field assistance. We thank two anonymous reviewers, whose comments improved the manuscript. All procedures were approved by the University of Utah Institutional Animal Care and Use Committee (protocol #08-08004). Funding was provided by NSF DEB-0816877 to DHC and the University of Utah Biology Undergraduate Research Program and Undergraduate Research Opportunities Program. JLW was supported by a University of Utah Graduate Research Fellowship. The authors declare that they have no conflict of interest.

## References

Ahmed FE, Mohammed A-HH (1978) *Haemoproteus columbae*: course of infection, relapse and immunity to reinfection in the pigeon. Z Parasitenkd 57:229–236

Anderson RM (1979) Parasite pathogenicity and the depression of host population equilibria. Nature 279:150–152

Anderson RM, May RM (1978) Regulation and stability of host-parasite population interactions: I. Regulatory processes. J Anim Ecol 47:219–247

Atkinson CT, Forrester DJ (1988) Pathogenicity of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. J Parasitol 74:228–239

Atkinson CT, van Riper C (1991) Pathogenicity and epizootiology of avian haematozoa: *Plasmodium, Leucocytozoon,* and *Haemoproteus*. In: Loye JE, Zuk M (eds) Bird-parasite interactions: ecology, evolution, and behavior. Oxford University Press, Oxford, pp 19–48

Blanchet S, Mejean L, Bourque J-F, Lek S, Thomas F, Marcogliese DJ, Dodson JJ, Loot G (2009) Why do parasitized hosts look different? Resolving the "chicken-egg" dilemma. Oecologia 160:37–47

Buchner A, Faul F, Erdfelder E (1997) G*Power: a priori, post hoc, and compromise power analyses for the macintosh (software), version 2.1.2. University of Trier, Trier

Cosgrove CL, Knowles SCL, Day KP, Sheldon BC (2006) No evidence for avian malaria infection during the nestling phase in a passerine bird. J Parasitol 92:1302–1304

Earle RA, Bastianello S, Bennett GF, Krecek RC (1993) Histopathology and morphology of the tissue stages of *Haemoproteus columbae* causing mortality in Columbiformes. Avian Pathol 22:67–80

Garvin MC, Homer BL, Grenier EC (2003) Pathogenicity of *Haemoproteus danilewskyi*, Kruse, 1890, in blue jays (*Cyanocitta cristata*). J Wildl Dis 39:161–169

Gregory R, Montgomery S, Montgomery W (1992) Population biology of *Heligmosomoides polygyrus* (Nematoda) in the wood mouse. J Anim Ecol 61:749–757

Hawlena H, Abramsky Z, Krasnov BR (2006) Ectoparasites and age-dependent survival in a desert rodent. Oecologia 148:30–39

Hochberg M, Michalakis Y, De Meeus T (1992) Parasitism as a constraint on the rate of life-history evolution. J Evol Biol 5:491–504

Hudson P, Dobson A (1997) Behavioural defence. In: Clayton DH, Moore J (eds) Host-parasite evolution: general principles and avian models. Oxford University Press, Oxford, pp 128–154

Hurlbert S (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54:187–211

Johnston RF, Janiga M (1995) Feral pigeons. Oxford University Press, New York

Kartman L (1949) Observations on the *Haemoproteus* of pigeons in Honolulu. Hawaii Pac Sci 3:127–132

Klei TR, DeGuisti DL (1975) Seasonal occurrence of *Haemoproteus columbae* Kruse and its vector *Pseudolynchia canariensis* Bequaert. J Wildl Dis 11:130–135

Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47:261–273

Marzal A, de Lope F, Navarro C, Møller AP (2004) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 142:541–545

McCallum H, Dobson A (1995) Detecting disease and parasite threats to endangered species and ecosystems. Trends Ecol Evol 10:190–194

Merino S, Moreno J, Jose Sanz J, Arriero E (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc Biol Sci 267:2507–2510

Paperna I, Smallridge C (2002) *Haemoproteus columbae* infection of feral pigeons in Singapore and Israel. Raffles Bull Zool 50:281–286

Perez-Tris J, Hasselquist D, Hellgren O, Krizanauskiene A, Waldenstrom J, Bensch S (2005) What are malaria parasites? Trends Parasitol 21:209–211

Sol D, Jovani R, Torres J (2000) Geographical variation in blood parasites in feral pigeons: the role of vectors. Ecography 23:307–314

Sol D, Jovani R, Torres J (2003) Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. Oecologia 135:542–547

Tomas G, Merino S, Moreno J, Morales J, Martinez-de la Puente J (2007) Impact of blood parasites on immunoglobulin level and parental effort: a medication field experiment on a wild passerine. Funct Ecol 21:125–133

Valkiūnas G (2005) Avian malaria parasites and other Haemosporidia. CRC Press, Boca Raton

van Oers K, Richardson DS, Sæther SA, Komdeur J (2010) Reduced blood parasite prevalence with age in the Seychelles Warbler: selective mortality or suppression of infection? J Ornithol 151:69–77

Yorinks N, Atkinson CT (2000) Effects of malaria on activity budgets of experimentally infected juvenile Apapane (*Himatione sanguinea*). Auk 117:731–738